CHARACTERIZATION OF SPROUTING OF *Cyperus rotundus* L. TUBERS UNDER FLUCTUATING TEMPERATURES

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ABSTRACT

Effects of fluctuating temperature on budbreak and shoot elongation, and the role of Ca^{2+} in bud break via heat pulse were determined in purple nutsedge (*Cyperus rotundus* L.) tubers

Dormant tubers were transferred to 35C for 30 minutes from 20C and 80% to 92% budbreak occurred. Tubers at 20C without the heat treatment had 20% to 25% budbreak. Even a 3-minute 35C pulse caused 62% budbreak. Budbreak following 35C for 12 hours was 92%, similar to that obtained with seven cycles of 20/35C (12/12 hours). A change in temperature from 25 to 15C or from 20 to 25C did not promote budbreak compared with constant 25 or 20C respectively. Varying the rate of temperature increase from 0.02 to 0.5C per minute in a single temperature shift from 20 to 35C had no effect on budbreak.

Tuber sprouts exposed to alternating temperatures with 12-hour 30C and 12-hour 40C elongated to 37 mm, 1.3 times higher than at constant 35C, an optimal mean temperature. Temperature differences of 2 and 4C in alternating cycle around the mean 24C had little effect on growth; an 8C differential had 21 mm greater shoot length than at constant 24C.

A single warm pulse of 35C for 1 h caused 60% to 70% budbreak of excised buds, and was substituted by ionomycin, and reduced by EGTA and verapamil. Excised buds without the single 35C pulse or with 1 mM verapamil had 36% to 42% budbreak. Suspension-cultured cells from purple nutsedge shoot tip loaded with fluo-3 AM resulted in cell expansion within 10 minutes. Intracellular $[Ca^{2+}]$ decreased or increased initially, then steadily increased with a 35C treatment, accompanied by the appearance of cytoplasmic strands. Withdrawal of heat stimulus did not cause a reduction of intracellular $[Ca^{2+}]$ in 30 minutes.

In conclusion, a single warm pulse stimulated budbreak of purple nutsedge, but not shoot elongation; the shoot elongation response was thermoperiodic. Ca^{2+} may mediate

the heat pulse-stimulated budbreak. The heat pulse also elevated the intracellular [Ca^{2+}] of cultured cells.

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2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ADP	adenosine diphosphate
AM	acetoxymethyl
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BA	benzyladenine
Captan	3a,4,7,7a-Tetrahydro-z-[(trichloromethyl)thio]-1H-isoindole-1,3
CTC	Chlorotetracycline
d	day(s)
DMSO	dimethyl sulfoxide
EGTA	ethyleneglycol-*bis*-(ß-aminoethyl ether) N,N,N',N'-1-teraacetic acid
ER	endoplasmic reticulum
g	gram
g	gravity
GA	gibberellic acid
h	hour
HEPES	N-(2-hydroxyethyl)pinperazine-N'-(2-ethanesulfonic acid)
Hsp	heat shock protein
HSR	heat shock response
IAA	indoleacetic acid
IP_3	ionsitol 1,4,5-trisphosphate
kDa	kilodalton

KT	kinetin
LSCM	laser scanning confocal microscope
MES	2-(N-morpholino)ethanosulfonic acid
min	minute
PCMBS	p-chloromercuribenzenesulfonic acid
PGR	growth regulator
picloram	4-amino-3,5,6-trichloropicolinic acid
PIP2	phosphatidylinosito 4,5-bisphosphate
PM	plasma membrane
PVP	Polyvinylpolyrrolidone
RH	relative humidity
RR	Ruthenium red
SE	standard error of mean
sec	second
TMB-8	8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate
TTC	triphenyl tetrazolium chloride

CHAPTER I

INTRODUCTION

Purple nutsedge (*Cyperus rotundus* L.) is considered the world's worst weed due to its wide distribution and infestation of agricultural lands (Holm et al., 1977). A major obstacle to the eradication of this perennial weed is the presence of dormant tubers and rhizomes in the soil, allowing its continual regeneration. Moreover, the buds have differences in the extent of dormancy; the population consequently exhibits sporadic release from dormancy and hence irregular sprouting. This temporal dispersal mechanism enhances the spread and survival of purple nutsedge.

The morphological structure of purple nutsedge provides a spatial advantage for its protection from many herbicides. The above-ground portion of the plant is a rosette of narrow grasslike leaves with, at maturity, a slender triangular culm bearing a reddish or purplish-brown inflorescence (Holm et al., 1977; Mercado, 1979; Wills and Briscoe, 1970; Wills, 1987). The underground portion is a complex and extensive system of rhizomes, tubers, and corms with a deep root system (Andrews, 1940; Ranade and Burns, 1925). The tubers are the principal means of purple nutsedge propagation (Justice and Whitehead, 1946; Muzik and Cruzado, 1953; Ranade and Burns, 1925; Rochecouste, 1956). At the base of the aerial shoot is a swollen corm or basal bulb that further connects with tubers (and parent tubers) through extensive rhizomes. Foliarly applied herbicide (glyphosate) is transferred from leaves to tubers (and parent tubers), and effectively kill them (Siriwardana, 1986; Zandstra and Nishimoto, 1977). Approximately 30 percent of the natural soil tuber population in the soil has no aerial connection (Siriwardana and Nishimoto, 1987). If dormant, they are not affected by applied glyphosate because of the absence of a direct connection. Subsequently, these dormant tubers resume sprouting and rapidly reinfest a field. Therefore, a strategy to control purple nutsedge must induce all

dormant tubers in the soil to sprout and create an aerial connection before treatment with a systemic herbicide such as glyphosate (Teo et al., 1973; Rehm and El-Masry, 1977; Doll and Piedtrahita, 1982).

The sprouting of purple nutsedge tubers can be effectively promoted by daily temperature alternations (Tripathi, 1967; Miles, 1991; Miles et al., 1994). Tuber sprouting in the field can be increased to near 100 percent through manipulation of soil temperature by soil solarization, and alternating temperatures of 20, 25, 30, 35, 40 and 45C (Miles, 1991). Importantly, exposure to 35C for 30 min per day with the rest at 20C is as effective as most of the alternating temperatures tested (Miles, 1991). However, their assessment of sprouting does not directly address tuber dormancy. First, the tuber was counted as sprouted when at least one shoot exceeded 1.0 cm in length (Miles, 1991), hence not separating the effects of temperature on the termination of bud dormancy from stimulation of shoot elongation. Second, all the experiments usually lasted at least 2 weeks, and the temperature was alternated daily . Hence, the number of cycles required to release the tuber from dormancy or for elongation and whether the thermal regulation of bud growth could be characterized as a rhythmic or switching behavior is unknown. Third, all the tubers used in the experiments were separated from a chain of tubers that exist in the field. Accordingly, whether alternating temperature impacted apical dominance within the chain(s) is not clear. The mechanism by which dormancy release occurs with temperature fluctuations is therefore not known.

It has long been known that alternating temperature breaks dormancy in seed (Lang, 1965; Thompson and Grime, 1977; Esashi et al., 1983; Steinbauer and Brigsby, 1957) and buds (Blake, 1972; Erez et al., 1979; Lyr et al., 1970; Powell et al., 1988; Stimart and Ascher, 1981; Downs and Bevington., 1981). However, the mechanism is not known. The transduction of this functional temperature signal is little documented although attempts to account for the effect of alternating temperatures on seed germination have

been made by Toole et al. (1955), Cohen (1958), and Esashi et al.(1983). Thus identification and characterization of the second messenger involved in thermal signaling would provide some insight to the understanding of the regulation and response of the thermal signal in breaking dormancy at the cellular and molecular levels. This study will contribute to the control of purple nutsedge as well as provide insights on signal transduction in plants.

The broad goal of this study is to understand the mechanism of alternating temperatures on stimulating tuber sprouting. The specific objectives are: First, to characterize the fluctuating temperature effect on budbreak; second, to determine the effect of various calcium channel inhibitors, calcium chelators, and ionophores on the warm pulse-inducible bud dormancy release of purple nutsedge tubers, and establish a relationship between the intracellular calcium signal and the warm pulse-inducible budbreak process; third, to determine whether changes in calcium ion levels of the cytosol occur in the purple nutsedge cultured cells as a result of the warm pulse treatment, and fourth, to characterize the fluctuating temperature effect on shoot elongation of purple nutsedge.

CHAPTER II

LITERATURE REVIEW

Dormancy Terminology

Numerous reviews on plant dormancy exist (Lang, 1987; Nooden, 1978; Romberger, 1963; Samish, 1954; 1964; Wareing, 1969; Martin, 1991; Aamlid, 1992; Egley and Duke, 1985; Perry, 1971; Hemberg, 1985). Over 50 terms are used to describe dormancy including the three main terms "dormancy", "quiescence", and "rest", and others with modifications based on (1) the seasons when dormancy occurs (i.e. summer dormancy, and winter dormancy), (2) the factors that caused the dormancy (i.e. photodormancy, thermodormancy), (3) the depth of the dormancy (i.e. deep dormancy, relative or conditional dormancy, correlative inhibition), (4) the various stages of dormancy (i.e. predormancy, early rest, middle rest, after-rest, postdormancy). To communicate precisely between scientists, Lang et al. (1985) defined " dormancy is the temporary suspension of visible growth of any plant structure containing a meristem." They incorporated specific physiological descriptors and proposed a dormancy terminology classified into eco-, para-, and endodormancy (Lang et al., 1985; Lang et al., 1987).

Purple nutsedge does not sprout below 10C (Orcutt and Holt, 1990; Shamsi et al., 1978; Tripathi, 1967; Ueki, 1969) or above 44C for a prolonged period (Orcutt and Holt, 1990; Shamsi et al., 1978; Tripathi, 1967; Ueki, 1969). Dormancy resulting from external temperature extremes is ecodormancy. If a covering structure such as a bud scale, causes a general microenvironmental condition that is unfavorable for growth of the enclosed meristematic tissues, the situation is analogous to unfavorable ambient environmental conditions; thus ecodormancy occurs. If the environmental cue is perceived by the bud scales and transported to meristematic tissue, this is paradormancy. "Correlation" events

such as "apical dominance," is another example of paradormancy. In contrast, if the environmental cue is perceived by the meristematic tissue , this is endodormancy. In most cases, dormancy is probably a complex combination of eco-, para-, and endodormancy since it is regulated both by environmental, various types of physiological factors, and regulatory events that occur gradually during the plant life cycle. For example, the unsprouted tuber in the field may be limited by a combination of apical dominance mediated by paradormancy and lack of temperature stimulus mediated endodormancy. Therefore, to classify each case of dormancy and apply the appropriate physiological term, a process-oriented hypotheses must be formulated and tested carefully with suitable experiments (Lang, 1987).

Our working definition of dormancy follows Lang's although the arguments for linguistics (Denney, 1989) or redefinding dormancy terms on a physiological basis (Junttila,1988) were considered. Several following special terms were used in this study:

Emerged bud--A bud in which the scale leaves grow out of the bud covering (scale) by macroscopic observation.

Sprouted tuber-- A tuber that has at least one visible emerged bud .

Fluctuating temperature--temperature rises and falls as wave(s), including daily alternating temperature and single warm pulse.

Daily alternating temperature--A daily step temperature change in a specific order (e.g. 12 h at 20C, 12 h at 35C) with no light treatment.

Single warm pulse--Temperature abruptly shifts from a low base temperature to a higher, but not extreme temperature, often for only a few minutes to two hours, then returns to the base constant temperature. The warm pulse also refers to heat pulse or thermal pulse.

Dormancy Termination by Fluctuating Temperatures

Characteristics of fluctuating temperatures on dormancy release. Fluctuating temperature is a complex of several variable factors: 1. Pre-conditioning with fluctuation temperature, 2. the rate of temperature change, 3. the upper or lower temperature achieved, 4. the temperature differential or amplitude, 5. the duration of upper or lower temperature exposed, 6. the number of temperature cycles, and, 7. the interaction with light (Bewley and Black, 1982).

Pre-conditioning with temperature fluctuations can determine the physiological stage of seeds or buds. Dry seeds usually need to be imbibed for a certain time before the cycles start. For example, storage temperature has been used to establish secondary dormancy in cocklebur (*Xanthium pennsylvanicum*) seeds (Esashi et al., 1983). Excising the bud or embryo is another way to obtain endodormant material (Teo et al., 1974). The rate of temperature change is an easily neglected parameter. This rate is gradual in the soil, and rapid in incubators or water baths. Lettuce seeds imbibed for 12 h at 22C, when exposed to different rates of warming to a maximum temperature of 30 to 34C had a similar germination occurred (Cohen, 1985).

The amplitude, or temperature differential can determine the efficacy of alternating temperature. In some species (*Typha latifolia*, *Apium graveolens*, and *Rumex sanguineus*), the magnitude of the fluctuation needs to be only a few degrees (Thompson et al., 1977; Thompson, 1974b). However, *Sorghum halepense* seed germination is stimulated by a temperature differential of 20C (15/35C), while 20/30C, and 23/37C did not enhance germination (Benech Arnold et al., 1990). Miles (1991) showed that the sprouting of purple nutsedge tubers increases with the increase in temperature differential. However, at temperatures greater than 40C, sprouting decreases.

The optimum exposure time for *Rumex* spp. seed to the upper temperature appears to be less at higher temperatures. For instance, the optimum is 30 to 40C for 1 to 4 h and at 45C only 30 minutes (Totterdell and Roberts, 1980). Moreover, the periods spent at each temperature obviously affect the efficacy of the amplitude (Totterdell and Roberts, 1980). If the higher temperature is applied to *Rumex* for 16 h/day, then an amplitude of at least 10C is required to obtain 90 % germination, whereas when the higher temperature is for only 8 h/day, an amplitude of ca. 5C is sufficient. By contrast, a minimum duration at 35C may be required for purple nutsedge tuber sprouting; tubers exposed to 35C for 30 minutes per day and the remainder at 20C gave almost the same stimulatory effect as the daily alternating temperature (12 h at 35C, 12 h at 20C) (Miles, 1991; Miles et al., 1994).

The induction of germination or sprouting increases to a maximum with the number of cycles of alternating temperature (Totterdell and Roberts, 1980; Benech Arnold et al., 1990). Repeated cycles of a particular regime are additive, each cycle resulting in the release from dormancy of a further proportion of the population (Benech Arnold et al., 1990). Under some conditions, a single fluctuation in temperature has nearly the same qualitative and quantitative effects as several temperature alternations (Taylorson and Hendricks, 1972a).

Whether upward or downward shifts are effective depends on the species. The efficacy of downward shift (pulse) is observed in *Amaranthus retroflexus*. Seeds do not respond to red light (i.e. to the P_{fr} produced) at temperatures above 35C. However, if light-exposed seeds are transferred to temperatures less than 32C for 2 h, the P_{fr} becomes effective and dormancy is released (Hendricks and Taylorson, 1978). Lateral *Ribes nigrum* (Tinklin and Schwabe, 1970), and *Oxalis* spp. bulbs show a similar response (Chawdhry and Sagar, 1974).

Shifts to a higher temperature, often for only 2 h, break seed dormancy of several species (e.g., *Nigella, Rumex , and Echinochloa crus-galli* (Isikawa, 1957; Vicente et al.,

1968), and some buds of *Iris* spp. (Halevy et al., 1964) in the dark. In most cases, however, the shift combines with the action of light to promote termination of dormancy and induction of germination. A 2 h period at 35C at the end of a 24 h dark imbibition period at 20C greatly enhances germination of *Lepidium* and other Cruciferae seeds in response to light (Toole et al., 1955, 1957). The 35C period is more effective when the red irradiation comes after rather than before the high temperature treatment (Toole et al., 1955), indicating that there is some interaction between P_{fr} and the temperature shift. Moreover, a distinct action of temperature has been recognized. A brief temperature shift (warm pulse) promoted germination of *Rumex* and other seeds in darkness (Taylorson and Hendricks, 1972a). The enhancement of germination by a single, brief temperature (20/40/20) shift (warm pulse) takes place rapidly during 8 to 16 min., and reaches its maximum in 1 to 2 h. Two treatments of a temperature pulse and red light applied together is more effective than the additive effects of the two applied separately.

In addition to the interaction between temperature and light, nitrate and oxygen have been reported to affect the fluctuating temperature effects. It has been reported that alternating temperature is the most efficient remedy, followed by light and KNO_3 to overcome the dormancy of seeds of grassland plants (Williams, 1983; Nelson, 1927). There is a synergistic action of temperature shift and imbibed in KNO_3 (0.2%) in breaking dormancy of light requiring rough cinquefoil seeds (Taylorson, 1969). The combination of the temperature shift plus nitrate increases the sensitivity to red light. Far-red light is, in itself, slightly promotive as small amounts of P_{fr} (ca. 2%) are sufficient to trigger the gemination of some seeds (Bewley and Black, 1982).

Immersion of plant buds in warm-water baths (30-40C) had long been used to break dormancy (Nooden and Weber, 1978). Boresch (cf. Nooden and Weber, 1978) reported that high air temperature (30C) combined with reduced air pressure (50 mm Hg) could break dormancy in plants not submersed, however high water temperature combined with

increased air pressure over the water to increase the oxygen concentration could not break dormancy in submersed plants. Molisch (cf. Nooden and Weber, 1978) found that neither high air temperature nor submergence alone could break dormancy.

In summary, the alternating temperature effect is a distinct phenomenon. Generally, fluctuating temperatures are an absolute requirement for dormancy release, while the order, rate of temperature changes, the temperatures involved, and time in each phase are not critical and vary with species.

Proposed models. The alternating temperature and pulsing temperature can be considered as two distinct stimuli although they might produce similar promotion at breaking dormancy. In the release of seeds or buds from dormancy, the alternating temperature acts as a rhythm or thermoperiodicity; the effect of each alternating cycle being additive. By contrast, a single temperature pulse may act as a trigger or a switch; once open, the germination or sprouting process occurs. Since the actions of alternating temperature and a single pulsing temperature are different, the physiological mechanisms could be different. The explanation of the temperature pulse effect therefore, may not be the same as for the alternating temperature effect. (Thompson, 1974a).

Very few attempts have been made to identify the mechanisms controlling responses to alternating temperature. Toole et al. (1955) have suggested that changes in temperature act biochemically, to alter the concentrations of reactants on which germination depends, perhaps through mechanisms involving enzyme thermodynamics. Cohen (1958) has proposed an explanation based on physical change of state correlated with the maximum temperature reached in the higher of the two temperature phases.

Several hypotheses have been proposed to explain the positive effect of alternating temperature. The earliest was advanced by Liebenberg (1884, reference see Aamlid,1992) and still seems quite plausible. Elevated respiration during the high temperature period is essential to provide metabolites required for embryo growth during the low temperature

phase. Furthermore, Toole et al.(1956) suggested that since different biochemical reactions within the seed have different temperature optima, a daily alternation of temperature might be required to attain a favorable balance of the various steps leading to germination. Later, Esashi et al. (1983) demonstrated that the cool phase triggered active engagement of the alternative respiration path during the subsequent warm phase, and this led to an increase in the ratio of the alternative path flux to the cytochrome path flux. The cool and warm phases therefore have different functions in adenylate metabolism. The former acted mainly in the production and accumulation of ATP, whereas the latter supplied ADP and, especially, AMP. Thus an increasing number of sequential alternating temperature cycles enlarged the size of the adenylate pool and the energy charge, both of which may be necessary for germination to take place. This model fits the additive property of alternating temperature. It should be noted that the material used in the experiment was secondarily dormant seeds. This mechanism might not be fully suitable for primary dormancy, although the fundamental biochemical or physiological distinction between the two has not yet been recognized.

Unlike alternating temperature, the response of the temperature pulse is a short-term regulation, and the temperature transition acts as a trigger. Lang (1965) has suggested that temperature changes are effective not because they remove specific inhibitors responsible for dormancy but because they increase the general physiological activity of the seed. To date, most of the hypotheses involved in the mechanism of the temperature pulse focus on the interaction between temperature and light. Single temperature pulse has substituted for or enhanced the light-induced breaking of dormancy mediated by phytochrome, indicating that temperature and light may break dormancy by the same mechanism (Bewley and Black, 1982). In the light-required seeds, the increases in germination caused by a brief high temperature pulse are due to the action of preexisting P_{fr} (Taylorson and Hendricks, 1972a; 1972b), while in the stimulation of dark germination

of *Rumex* species (Hand et al., 1982), the temperature pulse apparently increases sensitivity to preexisting P_{fr}. Several results by Taylorson and Hendricks (1972a) indicate that both stimuli, brief high-temperature periods and brief irradiations, act at or near the same site-a cell membrane or membranes which does not require protein or RNA change, and both may affect membrane permeability that might follow alterations in the passage of ions and metabolic status of the seed or bud, and terminate dormancy (Bewley and Black, 1982). However, no experimental evidence which bears directly upon these possibilities has been established, and the thermal signal transduction in the regulation of budbreak or seed germination is still a "black box".

The Role of Calcium in Dormancy Release and Germination

Calcium as a second messenger in plants. Dormancy-breaking and germination could be imposed by hormonal and environmental stimuli such as temperature and light. These primary signals evoke a series of cellular changes in biochemistry, metabolism and gene regulation, and finally causes morphological and physiological changes (Charkrabarti and Jenkins, 1987; Bewley and Black, 1982; Johnson et al., 1994). To link external signals and physiological responses, plants must have mechanisms to sense and transduce these signals. Anything that conveys information about the state of the plasma membrane to the cytoplasm are considered as a secondary messenger (Blowers and Trewavas, 1989).

Research during the last 15 years has established that Ca^{2+} acts as a second messenger in signal transduction in plants (Poovaiah and Reddy, 1993; Bush, 1993). Four criteria were proposed for evaluating whether Ca^{2+} is a messenger in regulating the physiological processes evoked by primary stimuli (Gilroy et al., 1991; Hepler and Wayne, 1985; Jaffe, 1980; Poovaiah and Reddy, 1987, and Poovaiah and Reddy, 1993). (1) Cytosolic Ca^{2+} must change in response to primary stimuli and such change should precede the

physiological response; (2) artificial induction of changes in cytosolic Ca^{2+} should evoke a physiological response in the absence of primary stimuli; (3) cells must possess the mechanism to sense the changes in cytosolic Ca^{2+} and translate them into a physiological response; and (4) blocking changes in the cytosolic Ca^{2+} or Ca^{2+} -sensing system must prevent the physiological response to external stimuli. Many plant cellular processes such as polarized growth (Lehtonen, 1984), mitosis (Welsh et al., 1978; Kiehart, 1981), cytoplasmic streaming (Tominaga et al., 1983), circadian leaf movements (Toriyama and Jaffe, 1972), guard cell swelling (McAinsh et al., 1992) were shown to be regulated by calcium ions. Calcium ions play a major role in various cellular processes (Roberts and Harmon, 1992).

The concentration of unbound cytoplasmic Ca^{2+} is usually very low (ca. 10^{-7} M), while the vacuole and the cell wall space calcium concentration are on the order of 1,000 to 10,000 times greater than that of the cytosol (Felle, 1988; Bush et al., 1989). The large gradient for calcium across most cellular membranes and into the cytosol , therefore, is potentially useful for amplifying signals into a regulated increase in cytosolic Ca^{2+}. A large influx of Ca^{2+} inside the cells may lead to death by activating enzymes such as lipases (Macknight, 1984), by perturbing general regulatory mechanisms normally controlled by Ca^{2+} (Berridge, 1985), by accumulating to toxic levels into intracellular organelles such as the mitochondria (MacKnight and Penniston, 1984), or by precipitation of calcium phosphate (Felle, 1988). For this signaling mechanism to work, therefore, the concentration of Ca^{2+} in the cytosol must be kept low, and this is achieved by actively pumping calcium to the outside of the cell or into the vacuole or other intracellular calcium stores (Puteny et al., 1981). The sources of increased intracellular Ca^{2+} vary with different stimuli (Bush et al., 1993; Poovaiah and Reddy, 1993).

The amplification of an external, weak signal mediated by the second messenger is achieved through a well-organized signal transduction pathway. A change in the

intracellular Ca^{2+} concentration is sensed by Ca^{2+}-binding proteins called response elements, which in turn regulate the cellular metabolism leading to a physiological response. To date, two Ca^{2+}-binding proteins have been well characterized in plants: one is Calmodulin (CaM), another is Ca^{2+}-dependent and CaM-independent protein kinase. The Ca^{2+}-binding protein, with a high affinity for Ca^{2+} , has no enzymatic activity in the absence of bound Ca^{2+}. When the concentration of cytosolic Ca^{2+} rises, the Ca^{2+}-binding protein bind to Ca^{2+}, and becomes active and interacts with a wide range of regulatory enzymes in cells. In summary, Ca^{2+} acts as a simple on-off switch that conveys the signal from the cell surface to the metabolic machinery, eventually resulting in a physiological response (Poovaiah and Reddy, 1993).

Involvement of calcium in germination and bud emergence. Calmodulin is considered to be a multifunctional protein. More than a half-dozen calmodulin-regulated enzymes have been found in plants. These include plasma membrane-localized Ca^{2+}-ATPase (Dieter, 1984), nuclear NTPase (Matsumoto et al., 1984), NAD kinase (Allan and Trewavas, 1985; Roberts et al., 1986), and kinases of soluble and membrane-bound proteins (Polya and Chandra, 1990). All of these enzymes play central roles in the regulation of various metabolic activities in plants.

Recent research showed that CaM-binding proteins may play a role in growth and development. It has been reported that the levels of CaM-binding proteins changes during the embryogenesis of carrot (Oh et al., 1992). Coccuci and Negrini (1988) found that an inhibitor of Ca^{2+}-CaM-dependent brain phosphodiesterase is present in the soluble fraction of embryo axes from ungerminated radish (*Raphanus sativus* L.) seeds, and the substantial decrease in the inhibitor is accompanied by an increase in CaM level during the first 24 h of germination. It appears that the balance of CaM and the inhibitor of Ca^{2+}-CaM complex controls the dormancy termination and the germination initiation of radish seeds.

There is also indirect evidence suggesting that calcium ions may be involved in seed germination and bud emergence. Davies and Millard (1985) studied the fractionation and distribution of calcium in sprouted and non-sprouted potato tubers using autoradiograph techniques. More than 90 percent of Ca^{2+} in the non-sprouted tuber could be considered to be in a physiologically active form. The concentration of ^{45}Ca in the sprouts increases with time. Sprouts that developed sub-apical tip necrosis showed a positive gradient of ^{45}Ca from the tip to the base, suggesting that Ca^{2+} is required for the sprouting of potato tubers. There are a few reports that addition of Ca^{2+} stimulates the budbreak of many plant, including 'Niagara Rosada' grapes (Pires et al., 1985), kiwifruit (Blank et al., 1991), potato (Ladyzhenskaya et al.,1991). However, the role of the applied Ca^{2+} in the process of budbreak or germination is unknown.

The interaction of cytokinin and calcium ions in bud formation from the protonemata of the moss *Funaria* supports the hypothesis that mitotic regulation by cytokinin may be due, at least in part, to modulation of intracellular calcium ion concentration. Cytokinin is ineffective in inducing moss bud formation in calcium-free medium, and the calcium ionophore A23187 can substitute for cytokinin in initiating bud development (Saunders and Hepler, 1982). The calcium ionophore A23187 increases the cytosolic calcium concentration by exchanging external Ca^{2+} for internal hydrogen ions. The increase in the concentration of intracellular calcium is correlated with moss bud initiation. It is worth noting that buds initiated by treatment with calcium and A23187 are unable to develop normally in the absence of cytokinin. This indicates that calcium ions may act as a second messenger, transforming the hormonal signal into a biochemical switch that regulates the initial stages of bud formation, and further development of the bud almost certainly involves changes in protein synthesis, possibly via cytokinin-induced alterations of transcription, RNA stability, or translation.

Red light initiates many important morphogenetic responses including dormancy breaking and gemination in plants through the mediation of the pigment, phytochrome. Roux et al.(1981, 1986) realized that the stimulating effect of P_{fr} on germination acts through the Ca^{2+}-calmodulin system. The following evidence supported this hypothesis. (A) In *Onoclea* spores, the P_{fr} form of phytochrome induced an increase in the intracellular Ca^{2+} concentration at an early step in the transduction chain and Ca^{2+} was considered to be a second messenger of phytochrome action. Two phenothiazine drugs (inhibitors of calmodulin) inhibited germination of *Onoclea* spore (Wayne and Hepler, 1984). (B) Non-green fern spores of *Lygodium japonicum* did not germinate in darkness. Germination was induced by red light through the phytochrome system (Furuya, 1983). Kagawa and Sugai (1991) suggest that red light induced the biosynthesis of gibberellin via the phytochrome system, and that gibberellin induced spore germination in *Lygodium*, Ca^{2+} was required during the germination process during the period following gibberellin synthesis. (C) Ca^{2+} participates in the early events of signal transduction in red-light-stimulated germination of other non-green *Dryopteris palaeaces* spores (Scheuerlein et al., 1989) and *Adiantum capillus-veneris* spores (Iino et al., 1989).

Involvement of Ca^{2+} in thermal signal transduction. A transient burst of cyosolic Ca^{2+} concentration has been reported in response to various environmental and hormonal signals such as wind (Knight et al., 1992), touch (Knight et al., 1991), wounding (Knight et al., 1993), gravity (Lee et al., 1983; Sievers, 1991), light (Gilroy et al., 1991; Gehring et al., 1990a), elicitor (Kauss and Jeblick, 1991; Knight et al., 1991), mechanical (Knight et al., 1991), anoxia (Subbaiah et al., 1994), redox (Price et al., 1994), ABA (Gilroy et al., 1990), salinity (Lynch et al., 1989), and IAA (Cohen and Lilly, 1984). Over 150 calcium-binding proteins have been identified and characterized, suggesting the significance of calcium in regulation of cellular metabolism (Moncrief et al., 1990). Temperature is an

exceedingly important environmental stimulus, and calcium may also act as a second messenger in thermal signaling pathway.

The effects of temperature-pulses on cytosolic calcium have been studied using transgenic tobacco seedlings that express aequorin (Knight et al., 1991). Seedlings rapidly transferred from a base temperature of 20 to 50C did not show any apparent changes in cytosolic Ca^{2+}, while cold-shocks to 0 and 5C induce rapid and transient increases in cytosolic Ca^{2+}. In animal cells, heat alters cellular Ca^{2+} homeostasis, inducing Ca^{2+} influx into the cytoplasm from both internal stores and the extracellular medium (Calderwood et al., 1988; Drummond et al., 1986). In plants, the phosphorylation of threonine residues of heat shock protein Hsp70 is dependent on calcium ions in vitro (Vidal et al., 1993). External calcium or heat shock greatly increase the expression of the calmodulin-related touch (TCH) genes of *Arabidopsis* (Braam, 1992). Recently, Ling et al. (1994) found that external calcium stimulated the production of a heat shock-related, calmodulin-activated enzyme, glutamate decarboxylase of fava bean roots. In addition, Harrington et al. (1996 personal communication) found that a rapid (15 seconds) heat shock induces increases in cellular inositol triphosphate (IP_3) in sugarcane cells. The elevated IP_3 proved to be capable of triggering Ca^{2+} release from intracellular storage compartments in animal cells (Calderwood et al., 1988). Accumulated evidence suggested that plant systems may share the same second messenger in the thermal signal transduction pathway as animal systems. There is no direct evidence that shows Ca^{2+} mediated heat-shock stimulus in plant systems.

Pharmacological Studies on the Establishment of Ca^{2+} Involvement in the Stimulus-Response Coupling

The Ca^{2+} movement between cytosol and extracellular solution or intracellular Ca^{2+} stores such as vacuole, endoplasmic reticulum (ER), and mitochondria is achieved through different transporters on the membranes in response to stimuli or to maintain a low cytosolic Ca^{2+} concentration. Many pharmacological agents that bind and block or antagonize specific transporters in plant cells inhibit the Ca^{2+} influx or efflux and/or cellular responses to stimuli (Takagi and Nagai, 1988; Lew el al., 1990). Therefore, if Ca^{2+} is a messenger involved in the signal transduction, blockage of the natural Ca^{2+} increase should inhibit the response in the presence of a stimulus (Jaffe, 1980).

Inhibition of Ca^{2+} influx. Channels are proteins in membranes to facilitate diffusion of ions such as Na^+, K^+, Ca^{2+} and Cl^-. Most of these ion channels are gated; that is, they are controllable and exist in open or closed states. Three types of gated ion channels have been detected in animal systems. One, the voltage-gated channel, opens or closes in response to changes in membrane voltage or electrical potential. In most cases, the voltage changes result from alterations in the distribution of ions or charged molecules on either side of the membrane. On the basis of their electrophysiological and pharmacological properties, the voltage calcium channels are classified into three subtypes. They are low threshold inactivating (T), dihydropyridine-sensitive, high-threshold noninactivating (L), and low threshold inactivating (N). The second type, the ligand-gated channel, opens or closes in response to binding specific control molecules. The third type of channel is the mechano-sensitive or stretch-activated channel, in which mechanical stresses on the membrane cause conformational changes that open the channel gates. All three types of calcium channels have been found in the plants (White, 1994; Huang et al.,

1994; Cosgrove and Hedrich, 1991; Millet and Pickard, 1988; Ding and Pickard, 1993a; Ding and Pickard, 1993b).

There are three major classes of organic Ca^{2+} channel blockers or antagonists, represented by the phenylalkylamines, e.g. verapamil, and D600; the benzothiazipines, e.g. diltiazem; and the dihydropyridines, e.g. nifedipine, nimodipine, and nisoldipine (Lee and Tsien, 1983; Harvey et al., 1989). All three types of calcium-channel antagonists modulate L-type voltage-sensitive calcium channels by binding to different sites in animal cells. Calcium channel blockers such as verapamil and diltiazem are tertiary amines, they attach to the lipid soluble portion of the plasma membrane, while nifedipine, which is uncharged at physiological pH (Lee and Tsien, 1983), acts at or near the plasma membrane surface (Takagi and Nagai, 1988; Bae et al., 1989).

Diverse organic drugs may show varying effects on certain calcium channel. For example, nifedipine is effective in the cessation of red light induced Ca^{2+} streaming in *Vallisneria* mesophyll cells, while verapamil is ineffective (Tsutsui, 1987). Similar results have been reported on the electrical properties of the cell membrane in the freshwater alga *Chara* (Tsutsui, 1987). In contrast, verapamil inhibits Ca^{2+} influx into carrot (*Daucus carota*) protoplasts (Graziana et al., 1988), and cucumber (*Cucumis sativus*) hypocotyls (Jackson and Hall, 1993). Also, it has been suggested that a single class of verapamil binding sites are present in maize (*Zea mays*) coleoptile membranes (Harvey et al., 1989). On the other hand, There are differences among sorghum (*Sorghum bicolor*) cultivars in response to verapamil, diltiazem and nifedipine (Wilkinson and Duncan, 1993). These data indicate that the sensitivity of each kind of calcium channel may vary among species, cultivars, or tissues.

Lanthanum (La^{3+}) is proposed as an inorganic Ca^{2+} channel blocker. Cytokinin-induced bud formation in *Funaria* is inhibited with both verapamil and La^{3+} (Saunders and Hepler, 1983). The inhibitory effect of La^{3+} on Ca^{2+} influx into the cytosol of *Vallisneria*

mesophyll cell protoplasts is similar to the effect of nifedipine (Takagi and Nagai, 1988). However, La^{3+} has no effect on verapamil-reversed auxin-induced elongation of cucumber hypocotyls (Jackson and Hall, 1993). The binding site for La^{3+} is still unknown. Lee and Tsien (1983) used electrophysiological techniques to examine the effects of organic and inorganic Ca^{2+} channel blockers on Ca^{2+} fluxes in cardiac cells. They proposed that the binding site for inorganic cadmium (Cd^{2+}) is different from organic blockers. Gadolinium (Gd^{3+}), a trivalent lanthanide, has no effect on voltage-gated calcium channels, but strongly inhibits stretch-activated channels (Morris, 1990). Millet and Pickard (1988) reported that Gd^{3+} strongly blocked stretch-activated-channel-involved gravity sensing in maize roots, while identical application of La^{3+} did not inhibit the tropisms noticeably. ω-Conotoxin has been identified as a specific antagonist for calcium N-type channels (Jones et al, 1994). These specificities of different channel blockers might have general utility for discriminating different types of Ca^{2+}-permeable channels.

Another way of inhibiting Ca^{2+} influx is to minimize extracellular free Ca^{2+} using divalent ion chelator. EGTA (ethylene glycol-bis-(b-aminoethyl ether) *N,N'*-tetraacetic acid) is the most commonly used extracellular Ca^{2+} chelator. The effect of EGTA on Ca^{2+} -mediated physiological response is quite concentration-dependent. EGTA inhibited the auxin-induced elongation of cucumber hypocotyls at high concentration, but stimulated the growth at low concentration (Jackson and Hall, 1993). Chlorotetracycline (CTC) is a more effective Ca^{2+} chelator than EGTA (Jackson and Hall, 1993).

In contrast to many PM calcium-channel blockers, TMB-8 (8-(*N,N*-diethylamino) octyl-3,4,5-trimethoxybenzoate) has been used in many tissues as an intracellular Ca^{2+} antagonist (Palmer et al., 1992). TMB-8 differentiates between dependence on intracellular and extracellular Ca^{2+} as it blocks efflux of Ca^{2+} from intracellular stores without affecting influx of extracellular Ca^{2+} (Palmer et al., 1992). So far, the plant cell studies indicate that TMB-8 acts by maintaining intracellular storage compartments of

bound Ca^{2+} (Schumaker and Sze, 1987; Saunders and Jones, 1988; Saunders and Hepler, 1983; Bourbouloux et al., 1992; Colorado et al, 1991; Moysset and Simon, 1989; Brummell and Maclachlan, 1989; Lew et al., 1990; Shetty et al., 1986). Combination of calcium-channel blockers for PM and intracellular stores might provide some indication of the source(s) of elevated cytosolic calcium in response to external signals.

Inhibition of Ca^{2+} efflux. To maintain the resting cytosolic Ca^{2+} at low concentration and to prevent Ca^{2+} ion becoming toxic, the extra cytosolic Ca^{2+} is effluxed through at least three pathways: P-type ATPases found on the PM , ER, and possibly, chloroplast membranes; Ca^{2+}/nH^{+} antiprotons on the tonoplast membrane, and uniports/channels on the plastid membranes (Bush, 1993). Among them, the P-type Ca^{2+} transporters are ATPase dependent, and have been widely studied with pharmacological agents. PCMBS (p-chloromercuribenzenesulfonic acid), a membrane impermeable sulfhydryl inactivator, has been used to inhibit ATPases through denaturing the sulfhydryl groups of the plasma membrane exofacial ATPases (Hedrich and Schroeder, 1989; Wilkinson and Duncan, 1993). Ruthenium red (RR) acts as a specific inhibitor for Ca^{2+} ATPase preventing the Ca^{2+} efflux from the cytosol and reversing many Ca^{2+}-mediated physiological responses to stimuli in plants (Wilkinson and Duncan, 1993; Jackson and Hall, 1993; Knight et al., 1992). Similarly, vanadate or erythrosin B as an another group of ATPase inhibitor (Bowman et al., 1978; Cantley et al., 1978) has been employed to pharmacologically characterize P-type transporter and study the Ca^{2+} messenger systems (Takagi and Nagai, 1988; Buckhout, 1983; Bush et al., 1989; Briskin, 1990; Bush et al., 1993). The Ca^{2+}/nH^{+} antiporter is dependent on the activity of H^{+}-ATPase, that is inhibited by nitrate (Bush and Sze, 1986; DuPont et al., 1990). The proportion of the two types of Ca^{2+} efflux transporters can be determined with separately applying vanadate and nitrate (Bush et al., 1993).

Promotion of cytosolic Ca^{2+}. In addition to the inhibition of Ca^{2+} movement by "antagonists", promotion of Ca^{2+} influx by calcium-channel agonists or calcium ionophores have also been applied to establish the involvement of Ca^{2+} in the signal transduction. L-type voltage-sensitive calcium channels can be activated by calcium - channel "agonists" such as BAY K 8644 in animal cells (Hess et al, 1984). A23187 is a divalent cationic ionophore as it is predominantly selective for divalent over monovalent ions. The selectivity sequence for divalent ions is $Mn^{2+}>Ca^{2+}>Mg^{2+}>Ba^{2+}$. It is capable of tranporting Ca^{2+} across biological membranes by complexation-decomplexation reactions with Ca^{2+} on the interface of the membrane (Pressman, 1976). The ionophore A23187 is applied to substitute for many stimuli and stimulate various Ca^{2+}-dependent biological reactions without disrupting preexisting balances of Na^{+} and K^{+} (Saunders and Hepler, 1982; Saunders and Hepler, 1983; Lew et al., 1990; Jackson and Hall, 1993). Certainly, it also transports Mn^{2+} and Mg^{2+}, but no report has shown that gradients of these ions across the membranes is of importance in biological control. All data in the reviewed literature has shown that A23187 ionophore can replace the Ca^{2+}-dependent stimulus induced physiological responses, and reverse the effect of Ca^{2+} channel blockers or antagonists, indicating that the cytosolic Ca^{2+} is a messenger in linking the various environmental signals and cell responses. Recently, another divalent ionophore, ionomycin, with stronger Ca^{2+} selectivity than A23187 without fluorescence has been introduced in the quantitative measurement of cytosolic Ca^{2+} concentration with fluorescence microscopy (Minta et al., 1989). Ionomycin may also be applied in the pharmacological studies of Ca^{2+} signalling.

Problems. Two major problems arise during pharmacological studies in plants. One is the specificity of drugs. TMB-8 is proposed as an intracellular calcium antagonist (Brummell and Maclachlan, 1989). However, it shows calcium-independent effects in certain animal cells, and directly inhibits protein kinase C activity rather than Ca^{2+}

mobilization (Brummell and Maclachlan, 1989). It also inhibits phosphatidylchloline formation and stimulates synthesis of phosphatidylinositol, phosphatidylglycerol and phosphatidylserine without altering the resting cytosolic Ca^{2+} concentration (Palmer et al., 1992). Interpretation of experimental results using TMB-8 treatment should be made with caution. In addition, the uniformity of calcium channels between animals and plants is not clear. Antibodies developed for dihydropyridine and phenylalkylamine receptors from skeletal muscle do not cross-react with plant membrane, suggesting some structural differences between the receptors in plants and animals (Harvey et al., 1989). Most of pharmacological agents used in plant signal transductions are from animal studies. One should therefore be careful when these drugs are employed in plants.

Another problem related with pharmacological studies is the penetration of the agents into the walled cells and/or multiple cellular tissues. Owing to unique plant structures, presence of a cell wall and the large volume of vacuoles, the agents may not reach the target channel proteins in plant cells as easily as in animals. In order to increase the drug effect, one could increase the concentration of the agents or extend the treatment time. However, these may cause side-effects. Chelators can cause serious damage, and thus it is suggested that extracellular Ca^{2+} chelator (EGTA) be used at low concentrations (<1 mM) when the treatment was longer than 24 h (Hepler and Wayne, 1985). High concentration of inorganic compounds may cause cell responses due to additional electrical potential and chemical potential, rather than external stimulations.

The recommendation for pharmacological studies in plants are (1) to use several pharmacological agents to test one hypothesis; (2) to keep treatment time as short as possible; (3) to establish experiments with concentrations and time courses of drug exposure.

Measurement of Cytosolic Free Calcium in Plants with Laser Scanning Confocal Microscopy

Advances in techniques for measuring cytosolic free calcium in plant cells are allowing us to understand the role of calcium as a messenger in the transduction of external signals. Although pharmacological evidence suggests the involvement of Ca^{2+} in external stimuli signalling in many cases, only the direct measurement of changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) during the response period in living cells is crucial to demonstrate signal-response coupling via Ca^{2+}.

The study of signal transduction in plant cells is much more difficult than with animal cells. The main reason for this is the special structure of the plant cells (i.e., the cell wall, and the large vacuole of plants), and other properties such as the difficulty of isolations of single cells by mechanical and enzymatic methods, extracellular ester hydrolysis, or incomplete internal dye hydrolysis, dye compartmentalization, leakage, and toxicity (Bush and Jones, 1990; Williams et al., 1990; Cork,1985; Brownlee and Wood, 1986; Gilroy et al., 1987; Jackson and Hall, 1993; Poovaiah and Reddy, 1987, 1993).

The invention of calcium-sensitive fluorescent probes and the availability of powerful, low-cost computers that can be used for video image analysis has brought about a revolution in the measurement of Ca^{2+} in living cells. Ca^{2+}-binding fluorescent dyes much more commonly applied in the Ca^{2+} measurement of plant cells than other methods such as photoprotein (i.e. aequorin), Ca^{2+}-sensitive electrodes. When the new generation of dyes such as Quin-2, Indo-1, fura-2 and fluo-3 that have a high affinity and selectivity for free Ca^{2+} are applied with recently developed microscope technologies (i.e., fluorescence ratio imaging or laser scanning confocal microscopy (LSCM)), cytosolic free calcium dynamics in single cells can be quantified precisely (Read et al., 1992).

Advantages of LSCM. There are several distinct advantages of the LSCM over the conventional fluorescence microscope. First, the LSCM provides improved rejection of out-of-focus light arising from the excited fluorophore and capacity of performing an optical section through a thick tissue. In contrast, a conventional fluorescence imaging produces the unwanted contribution of signals from structures above and below the plane of focus, resulting in a background glow that can distort the image (White et al., 1987). Second, the resultant high vertical and horizontal spatial resolution of the confocal technique affords the ability to image Ca^{2+} within the context of detailed intracellular structure, and to measure changes in many cells and in neighboring tissues simultaneously and noninvasively (Gehring et al., 1990b). With a computer reconstruction program, a three-dimensional, or a rotational visualization of biological specimens and identification of sites of Ca^{2+} release can be achieved. Third, since the depth of illuminated sample is under control, and the volume of the particular part of the cell is measurable, it is possible to precisely obtain an absolute concentration of the intracellular Ca^{2+}. Fourth, the confocal imaging system is approximately 2.4 times more sensitive than direct photography, opening up the exciting possibility of being able to visualize the rapidly dynamic changes in $[Ca^{2+}]_i$ during the signaling process (White et al., 1987).

Instrumental properties. The most commonly used confocal microscope in visualization and measurement of the calcium message in plant cells is the laser scanning confocal microscope. The Bio-Rad MRC-600 LSCM system is equipped with a 25-mW argon-ion laser, and also with both single- and dual-detector. Images can be collected simultaneously in the two channels and are completely recorded with each other; this is ideal for dual-emission ratio imaging with suitable dyes. The strongest excitation lines (488 and 514 nm) are projected through a pinhole and focused by condenser lens to a diffraction-limited spot in the specimen. Emitted light is collected through a pinhole aperture. The pinhole provides the mechanism for the rejection of out-of-focus light. To

generate two-dimensional cell images, it is necessary to scan the laser beam across the specimen and record the single-point detector output in a two-dimensional array. Laser beam scanning can be done by vibrating mechanically positioned mirrors. With this scanning system, vertical spatial resolution of approximately 0.5 to 0.7 μm is obtained using 40x or 60x high numerical aperture objectives. The maximum rate of forming a full-frame cell image acquisition is about one frame per second, which is not sufficient to visualize intracellular Ca^{2+} dynamics of interest such as Ca^{2+} oscillations and wave propagation. This limitation can be improved with a rotating disk containing pinholes (Nipkow disk), and with a slit scanner (Diliberto et al., 1994). The confocal system is interfaced with a Research Machines Nimbus computer possessing an 80486 microprocessor and 110-Mbyte hard disk. Image archiving is achieved with a Panasonic 940 WORM optical disk drive utilizing 940-Mbyte capacity optical disks. The new mouse-driven, COMOS software is used to drive the confocal system and this software includes several basic analysis functions (such as generating a series of quantitative parameters (e.g., fluorescence intensity, area, perimeter, shape, and number of particles within the cell), making histogram and scattergram files, and analyzing single- and dual-color Z-series scans, and combining a series of 3-D images into a rotational "movie." Also, the THRUVIEW software is available for animate time courses of $[Ca^{2+}]_i$ dynamics in optical sections successively captured in a single image plane. Recently Kuba et al. (1994) developed a UV laser-scanning confocal microscope by modifying the optics of a conventional LSCM to allow imaging intracellular Ca^{2+}-dependent fluorescence with a UV laser (351 or 364 nm). This confocal microscopy yields a lateral resolution of < 0.3 μm and an axial resolution of < 1.5 μm and a relevant field size of 100 μm in diameter for a 40X objective. Latest LSCM, RCM-8000 Real-Time (Nikon Ins.) is capable of instantaneous acquisition and review of images at up to 30 frames per second with unsurpassed sensitivity, speed, optical sectioning, and resolution.

Ca^{2+} Selective Dyes. ***Types of dye.*** There are two major types of fluorescent Ca^{2+} indicator dyes currently available for use in confocal free Ca^{2+} imaging: (1) signal-wavelength or non-ratio dye such as fluo-3, rhod-2, Calcium Green, Calcium Orange, Calcium Crimson, and Fura-Red (Minta et al., 1989; Haugland, 1992), (2) dual-wavelength or ratio dyes such as fura-2 and indo-1. Single-wavelength dyes are excited by visible wavelength light and show no spectral shift on binding Ca^{2+}. When free Ca^{2+} increases, the fluorescence intensity may decrease (Fura-Red) or increase (all others) proportionately across the whole emission spectrum. However, the amount of fluorescence is dependent on the amount of dye measured, that can vary as a result of unequal distribution of dye within or between cells, and leakage and/or photobleaching of dye during an experiment. Therefore it is difficult to accurately calibrate the $[Ca^{2+}]_i$ using single-wavelength dyes.

On the other hand, all dual-wavelength or ratiometric dyes are excited by ultraviolet (UV) wavelength light with a resultant emission in the visible spectrum (Grynkiewicz et al., 1985), and exhibit Ca^{2+}-dependent spectral shift in either the excitation (fura-2), or emission (indo-1). The characteristics of these dyes permit determination of dual-wavelength ratio values that are dependent on the Ca^{2+} concentration but are independent of the intracellular dye concentration. Thus quantification of $[Ca^{2+}]_i$ is much more precise with dual-wavelength days.

Loading plant cells with calcium dyes. Loading plant cells with efficient and homogeneously distributed calcium dyes is always a very important step in the plant cell biology . The free dyes are hydrophilic, and do not cross the plasma membrane easily. Unique structural features of plant cells such as cell wall make it more difficult for dyes to penetrate plant cells. Four loading techniques have been used in Ca^{2+} confocal visualization and measurement. (1) ester loading (Gehring et al., 1990a and b; Williams et al., 1990), (2) microinjection of the cell-impermeant salt forms of dyes into single cells

(Franklin-Tong et al., 1993), (3) electroporation (Jackson and Heath, 1990), and (4) digitonin permeabilization (Timmers et al., 1991).

Ester loading is currently the most efficient and useful method for dye introduction. The acetoxymethyl (AM) esters of the Ca^{2+}-dyes are highly lipophilic and cross the plasma membrane easily. Once inside the cell, the esterified derivatives are cleaved by the endogenous cytosolic hydrolases, thus trapping the active free dye inside the cell. The method is much less labor intensive than microinjection and electroporation, and produces very reproducible results within a given cell population; these cells can be imaged quickly and simultaneously with minimal damage. Loading has been obtained by incubating cells for 20 min to 24 h in the presence of 1 to 50 μM of the dye-esters at temperatures of 4 to 30C (Read et al., 1992). Uptake of esterified fluo-3 can be enhanced in the presence of the low-toxicity detergent Pluronic F-127 (Molecular Probes, Inc.) with centrifugation at 15,000×*g* for 30 sec to 3 min (Gehring et al., 1990b).

Ca^{2+} binding affinity. The effect of chelation of Ca^{2+} by the intracellular dye molecules on the intracellular free Ca^{2+} concentration is another aspect in the dye selection. Values for dye-Ca^{2+} dissociation constants (Kd) vary with the various dyes. The Kds for Fura-Red, fluo-3, and rhod-2 are 133 nM, 316 nM, and 1.0 mM, respectively. Low Kd means high Ca^{2+} affinity. Sufficient quantities of high affinity dyes (low Kd Values) such as fura-2, indo-1, and Fura-Red, may buffer or "clamp" Ca^{2+} levels in the same range as resting intracellular Ca^{2+} (< or = 100 nM), and become saturated below micromolar concentrations of Ca^{2+}. This may reduce the sensitivity required for monitoring high magnitudes of Ca^{2+} alterations that may occur in subcellular regions (nucleus, neuronal plasma membrane) due to agonist stimulation of the artificial induced Ca^{2+} increases resulting from caged Ca^{2+} release. Several of the newer visible wavelength dyes (fluo-3, rhod-2, Calcium Green, etc.) have weaker affinities for Ca^{2+}, thereby improving the ability to monitor changes in higher Ca^{2+} levels (Diliberto et al., 1994).

In terms of dye loading and Ca^{2+} binding affinity, fluo-3 AM is a good candidate for the Ca^{2+} indicator dye. However, since it is not a ratiometric dye, its quantitative application has some limitations. An alternative to solving this problem is to co-load fluo-3 AM with Fura-Red/AM into the cells. Both Ca^{2+} dyes are excited with the 488-nm line of a standard argon ion laser, while emission is split to dual-channel photomultiplier tube detectors (525 nm for fluo-3, and 645 nm for Fura-Red). However, the Ca^{2+} binding affinity and distribution of the two dyes are not identical, thus the reliable ratio imaging of $[Ca^{2+}]_i$ has not been achieved (Diliberto et al., 1994). An ideal dye would be a dual-visible-wavelenth dye with properties of loading and Ca^{2+} binding affinity similar to fluo-3.

Calibration of cytosolic free calcium concentration. It was pointed out that calibration of cytosolic free calcium concentration can be useful for the quantification of cytosolic Ca^{2+} levels. However in most cases, particularly in a primary experiment, qualitative changes in ion concentrations and their relative spatial distribution within a single cell are the important concern (Read, et a., 1992; Franklin-Tong et al., 1993).

Calibrations for quantitative measurements of intrcellular Ca^{2+} values in living cells can be conducted *in vitro* or *in vivo*. Calibration *in vitro* is simple, but not critical because of subcellular environmental heterogeneities and unequal dye distribution in the cell. Calibration *in vivo* is more complicated. This is usually performed on the cell under study at the end of an experiment. It normally requires determination of the maximal (dye/Ca^{2+}) and minimal (free dye) fluorescence intensities within the cell. The maximal and minimal fluorescence intensity can be determined using a Ca^{2+} ionophore such as ionomycin to raise intracellular Ca^{2+} levels in the presence of a known extracellular Ca^{2+} concentration. The minimal fluorescence intensity can be accomplished by sequential treatment of cells with heavy metals (Mn^{2+} or Zn^{2+}) to quench Ca^{2+}-dependent fluorescence, and by release of intracellular dye by digitonin. $[Ca^{2+}]_i$ is then calculated from the equation $[Ca^{2+}]_i$ =

Kd(F-Fmin)/(Fmax-F), where Kd is approximately 400 nM and the fluorescence enhancement of fluo-3 on Ca^{2+} binding is ca. 36- to 40-fold (Minta et al., 1989). *In vivo* calibration of fluo-3-loaded plant cells by these methods has been successfully achieved (Williams et al., 1990; Gehring et al., 1990b). Compared with calibration *in vitro*, calibratrion *in vivo* is more precise due to the consideration of the potential variations in intracellular environmental factors that may affect the spectral properties of the dye (i.e. local ionic strength, the presence of heavy metals, polarity, viscosity of the local environment). However, some limitations of this method should be taken into account. In many cases, it is difficult to equilibrate the Ca^{2+} concentration throughout multiple intracellular compartments of plant cells. Again, when applying low affinity Ca^{2+}-binding dyes (such as flou-3), increasing the $[Ca^{2+}]_i$ up to the point of dye saturation by addition of an ionophore with low Ca^{2+} selectivity may have trouble (Diliberto et al., 1994). Solutions to these problems may be to use the higher Ca^{2+} selectivity ionophore ionomycin, instead of A23187 or Br-A23187, and to lyse plant cells with a low concentration of digitonin or Triton X-100 (William, 1990).

CHAPTER III

THERMAL TRIGGERED DORMANCY RELEASE OF PURPLE NUTSEDGE TUBER BUDS

Introduction

Purple nutsedge is a perennial weed, and buds of purple nutsedge show variability in depth of dormancy. The sprouting of purple nutsedge tubers is strongly promoted by daily temperature alternations (Tripathi, 1967; Miles, 1991; Miles et al., 1996). Miles (1991) showed that it is possible to increase sprouting of individual tubers in the field to near 100% through manipulation of soil temperature by soil solarization. A daily 30 min exposure to 35C, followed by incubation under 20C appears to be all that is required to break bud dormancy (Miles et al., 1996). However, it is unclear if the multiple temperature alternations are necessary for budbreak. Understanding of this question and other attributes of the alternating temperature function (Totterdell and Roberts, 1980) is prerequisite to studying the mechanism involved in the thermal control of breaking dormancy.

The phenology of thermal breaking of seed or bud dormancy has been well documented (Lang, 1965; Thompson and Grime, 1977; Esashi et al., 1983; Erez et al., 1979; Powell et al., 1988; Stimart and Ascher, 1981; Downs and Bevington, 1981; Benech Arnold et al., 1990). Based on the characteristic of the functional temperature, thermal behavior could be classified as thermoperiodicity or a trigger. The thermoperiodic response occurs when dormant seeds or buds are exposed to alternating temperatures, thereby increasing germination or sprouting compared with the same mean, but constant temperature. This rhythmic action needs a number of continual alternating cycles, and the effect of each cycle is additive (Esashi et al., 1983; Probert et al., 1989; Benech Arnold et

al., 1990). By contrast, the thermal trigger response occurs when dormant tissues receive a single brief fluctuating temperature, such as a thermal pulse of a few min to 2 h in which temperature abruptly shifts from a low base temperature to a higher, but not extreme temperature, and return to the base temperature for the rest of experimental period. The trigger action is brief and need not continue once dormancy is broken (Amen, 1968). Shifts to a higher temperature, often for only 2 h, can break dormancy in darkness of some seeds of several species, e.g., *Nigella*, *Rumex*, and *Echinochloa crus-galli* (Isikawa, 1957; Vicente et al., 1968; Taylorson and Hendricks,1972a; Taylorson and Dinola, 1989), and some buds of *Iris* spp. (Halevy et al., 1964). In most cases, however, the shift combines with the action of light to promote termination of dormancy and induction of germination (Toole et al., 1955; 1957).

Previous work done on the effect of alternating temperature upon the sprouting of purple nutsedge tubers used the elongated shoot (> 1 cm) as a parameter for sprouting (Miles et al., 1996); however, that parameter may contain two thermal-regulated steps: dormancy release and elongation (Nishimoto et al., 1995). The work reported here concentrated on the dormancy release aspect. Budbreak was used as the criterion for tuber dormancy release. Experiments were conducted to determine the extent of budbreak to various parameters of fluctuating temperature, including the number of thermal pulses, rates of thermal shift, duration of warm treatment, the upward or downward shifts in temperature, and the magnitude of fluctuating temperatures.

Materials and Methods

Plant material. Purple nutsedge tubers were obtained from the fields at the University of Hawaii's Waimanalo Research Farm and grown in 11-liter pots containing potting medium in a glasshouse. The medium was composed (2:1:1) of peat moss: vermiculite: perlite, and

23 g (Osmocote 18-2.6-9.9) (Grace Sierra, Milpitas, Calif.)/pot (100 lbs/acre of N). The additional 15 g Osmocote/pot was applied with top-dressing 3 months after the planting. The pots were watered three to four times a day to avoid desiccation of extensive growth, especially for 4 to 6 month old plants. The soil temperature in the center of the pot had a sine-wave pattern with the minimum and maximum temperatures of 23 and 29C respectively. Eight sprouted tubers were initially planted in each pot, and 6 months later, the mature black or brown tubers were ready for the experiments.

Selection of dormant tubers. The entire pot of tubers were brought to the laboratory at constant 20C to avoid undesigned heat exposure. The unsprouted tubers were separated from the mother plants or sprouted tubers on the rhizome-tuber chain and trimmed of roots and rhizomes. Tubers were then allowed sprout in the dark on two layers of filter paper moistened with deionized water in 15 cm Petri dishes enclosed in a clear polyethylene bag to conserve moisture. After incubation at 20C for 2 weeks, ca. 25% of the tubers remained unsprouted, (Nishimoto et al., 1995); the unsprouted tubers were considered dormant tubers, and used in the following experiments.

Evaluation of temperature effect. Ten unsprouted tubers were placed on two layers of filter paper moistened with 2 ml deionized water in 9 cm Petri dishes enclosed in a polyethylene bag to maintain moisture. The tuber was counted as a sprouted tuber when one or more emerged buds on a tuber were visible. Sprouting of tubers at constant 20C under the daily counting condition increased to approximately 20% of the total population at the final count. Therefore, the number of sprouted tubers was recorded only at the end of the experiments. The experiments lasted 7 d unless otherwise noted. The tuber was considered sprouted tuber when one or more buds emerged. Budbreak was based on the sprouted tubers over viable tubers in each Petri dish. For the test of tuber viability, the unsprouted tubers were incubated at alternating temperature of 20/35C, 12/12 h for 7 d; if tubers still remained unsprouted, they were treated with a triphenyl tetrazolium chloride

(TTC) as described by Miles et al (1996). In most cases, the total viability of the tuber population was ca. 100%.

Temperature treatments. The daily alternating temperature regime of 35/20C for 12/12 h was chosen based on their maximum stimulatory sprouting effect (Miles et al., 1996). The short period of temperature fluctuations (thermal pulses) were supplied by transferring tubers to incubators (±1.0C) for the required time intervals, then returning to 20C or 25C incubators in the dark. It took ca. 30 min for the tuber's surface temperature to reach 35C from 20C in the 35C incubator. A 3-min pulse of 35C was implemented by placing tubers at 55C for 3 min (time for the tuber surface temperature to reach 35C), then returning to 20C.

To evaluate the effect of temperature change rate, a sine-wave temperature curve was obtained by using dry sand as a heat buffer. The Petri dishes containing tubers on two layers of moistened paper were buried 15 cm below the surface of sand in a foam container (26x36x30 cm) holding 20 cm of dry sand. The foam container was transferred into an incubator in which the temperature shifted from 20 to 49C for 12 h, then to 6C for 12 h before returning to 20C. As a result, the tuber temperature gradually increased from 20C to 35C in 12 h, and then slowly decreased to 20C, and remained at 20C. The corresponding temperature shift rate was similar to those of soil temperature in natural conditions.

In a experiment with low temperature versus high temperature pulse, unsprouted tuber were selected after incubation at 25C for 2 weeks, and 25C was used as the base temperature, instead of 20C in all other experiments. Tubers were exposed to 15C for 12 h (low temperature) or to 35C for 12 h (high temperature) then returned to 25C. The number of tubers with budbreak was recorded at 5 days.

Statistical analysis. Each experiment was a completely randomized design, consisting of four replicate dishes with 10 tubers per treatment, and conducted at least twice. Results

between experiments were similar. The number of sprouted tubers in a Petri dish was transformed to percentage before analysis. Percent data were not binomially distributed, and were subjected to analysis of variance (ANOVA) without transformation to determine sources of experimental variation and provide the necessary estimates for calculating the standard errors of means (SE). Tuber response to number and duration of warm pulses were analyzed by nonlinear (exponential) regression. Duncan's multiple range test was used to determine whether treatments were significantly different.

Results

Transition of dormancy by a single temperature fluctuation. Only 25% of dormant purple nutsedge tubers sprouted when incubated at constant temperature of 20C for 7 d, while a single warm temperature pulse at 35C for 30 min dramatically increased budbreak three-fold more than the constant 20C regime (Fig. 3.1). The tuber sprouting response to the number of warm pulses (35C for 30 min) fitted an exponential curve (Fig. 3.1). The variance of sprouting in the single pulse was much larger than multiple pulses, but the budbreak mean for the single pulse was not significantly increased as the number of the pulses increased, suggesting that tubers were highly sensitive to a single fluctuation in temperature. It appeared that the effect of heat units (Deg C > 20C x Time (min)) on budbreak was not additive, since the first fluctuation was sufficient to cause dormancy release of buds.

Duration of thermal triggering. Although a single 30-min thermal pulse of 35C could provide equivalent tuber sprouting to seven cycles of the same pulse, 10% to 20% of the dormant tubers remained unsprouted. The unsprouted tubers were subjected to different durations of the 35C warm treatment in a single temperature fluctuation to determine if they needed a longer period of warm temperature for the budbreak process. Increasing

the durations from 30 min to 12 h at 35C provided a similar stimulation in budbreak, and the response of tuber budbreak to time at high temperature was exponential (Fig. 3.2). Although 30 min of incubation at 35C could effectively break dormancy, the tubers were at 35C for only a few min, as it took 30 min for the tuber surface to reach 35 C. These observations led us to further shorten the thermal phase by accelerating the shift in temperature to demonstrate the rapidity of the thermal behavior. Consequently, a 3-min thermal pulse was designed so the tuber temperature reached 35C within 3 min, and this also caused a three-fold increase in tuber budbreak over the constant 20C. Budbreak from the 3-min pulse was 25% lower than that of 30-min pulse (82%) (Fig. 3.2).

Independence of shifting rate. Transferring tubers from one incubator to another caused a rapid change in tuber temperature. The rate of temperature change was 0.5C per min, while the rate in the sand-buffered condition was 0.02C per min, approximately 24-fold less than the former. However, both shifts provided similar sprouting, close to the sprouting observed with daily alternating temperatures and significantly different from constant 20C (Fig. 3.3).

Low temperature versus high temperature pulse. Tuber budbreak at constant 25C was 55% (Fig. 3.4), significantly higher than at 20C. The comparison of a warm temperature shift (35C for 12 h) and a cold temperature shift (15C for 12 h) with the base temperature of 25C revealed that the stimulation of budbreak occurred only with the warm temperature shift, the cold temperature shift was ineffective (Fig. 3.4).

Effective magnitude of a single thermal shift. Both long-term (3 h to 12 h) and short-term (30 min) heat treatments were employed. The magnitudes of 5, 10, 15, and 20C were set by changing temperature from 20C to 25, 30, 35, and 40C respectively. To eliminate the influence of accumulative temperature on budbreak, a set of heat treatments with different exposure times from 4 to 12 h, but same heat units (3600C·min) were used.

A single shift of 5C showed no budbreak stimulation; only magnitudes of 10C or larger promoted sprouting. Increasing the peak temperature
from 30 to 40C did not significantly increase sprouting (Fig. 3.5, (a)). In the 30-min heat treatments, the sprouting at 30, 35, and 40 C pulses was greater than at constant 20C, and there was no significant difference among the heat treatments (Fig. 3.5, (b)).

Discussion

The dormancy transition is driven by many environmental stimuli such as dry storage, light, and temperature (Amen, 1968). The effectiveness of the stimuli depends on different plant species. For purple nutsedge, one shift in temperature from 20C to 35C for 12 h and reincubation at 20C for 6.5 d caused over 92% sprouting, and was equivalent to a 7-d temperature alternation (20/35C, 12 h each per day) (Fig. 3.3). Furthermore, a single 30-min exposure to 35C produced similar sprouting to seven cycles of the same temperature shift (Fig. 3.3). These results suggested that the first shift in temperature was sufficient for dormancy transition of tubers; the subsequent multiple shifts were not necessary for the budbreak. In fact, only a few min of high temperature (35C) could initiate dormancy release and cause budbreak (Fig. 3.2), indicating that recognition and reception of the thermal signal by tuber cells was very rapid, and that the transduction of the external thermal signal to intracellular chemical or biochemical messenger was irreversible. The 3-min thermal pulse had ca. 25% less budbreak than the 30-min pulse (Fig. 3.2). This may result from an insufficient temperature equilibrium when rapidly heating tubers in a few min (Taylorson and Hendricks, 1972b). Taken together, our current data suggested that the response of breaking dormancy to the warm temperature was a single and brief action like a trigger or a switch, rather than a rhythm.

The daily alternating temperatures consistently resulted in 100% budbreak (Fig. 3.3) and subsequent shoot elongation (Miles et al, 1996), dormancy of all single tubers therefore can potentially be broken by fluctuating temperatures. A single pulse of temperature from 20C to 35C for 30 min usually did not cause 100% sprouting within 1 week (Fig. 3.1 & 3.2). Incubation of thermal pulse-treated tubers at constant 20C for another 1 week gradually increased budbreak, but a few viable tubers remained unsprouted (data not reported). Addition of either 30-min multiple pulses, or prolonging the duration of high temperatures in a single shift alone could not cause 100% budbreak. However, a combination of increasing the number of temperature fluctuations and prolonging the duration of warm treatments (Fig. 3.3) may be the preferred conditions for dormancy release of tubers, and accelerating budbreak.

An upward shift in temperature from 25 to 35C for 12 h effectively induced the budbreak, while the downward shift from 25 to 15C for 12 h had no effect on the budbreak (Fig. 3.4), indicating that the warm phase shift in the fluctuating temperature was more important than cold phase shift. The crucial factor influencing the bud dormancy release was the magnitude of the upward shift. When the tuber temperature increased 10C or higher above the base temperature 20C, the thermal response occurred. This characteristic is similar to a heat shock response (HSR). The heat shock temperature is generally 8 to 10C above the "normal" growing temperature (Key, 1992). The temperature of 20C was considered a suitable condition for the purple nutsedge budbreak since about 75% budbreak occurs when freshly harvested tubers are incubated at constant 20 (Nishimoto et al., 1995). Our 15C temperature increase emulates condition reported to cause heat shock, and we suggest that heat shock proteins may be involved in the thermal signal transduction and developmental regulation of the dormancy release of purple nutsedge tubers.

The induction of budbreak by brief periods of exposure to 35C was in effect a trigger or switch in the dormancy control mechanism. Such sensitivity could explain how a tuber population collected from the field sprouts readily (up to 85%) when incubated at room temperature (Miles, 1991). The budbreak could be induced by exposure to high temperatures during the collection and handling process.

Brief exposures to an elevated temperature stimulated budbreak of tubers in the laboratory, and probably have an important role in triggering budbreak of purple nutsedge tubers in the field. In addition, the fact that the rate of temperature change was not critical for budbreak (Fig. 3.3) suggests that gradual increases in temperature that occur in natural soil could serve as the stimulus for budbreak of purple nutsedge tubers in the field.

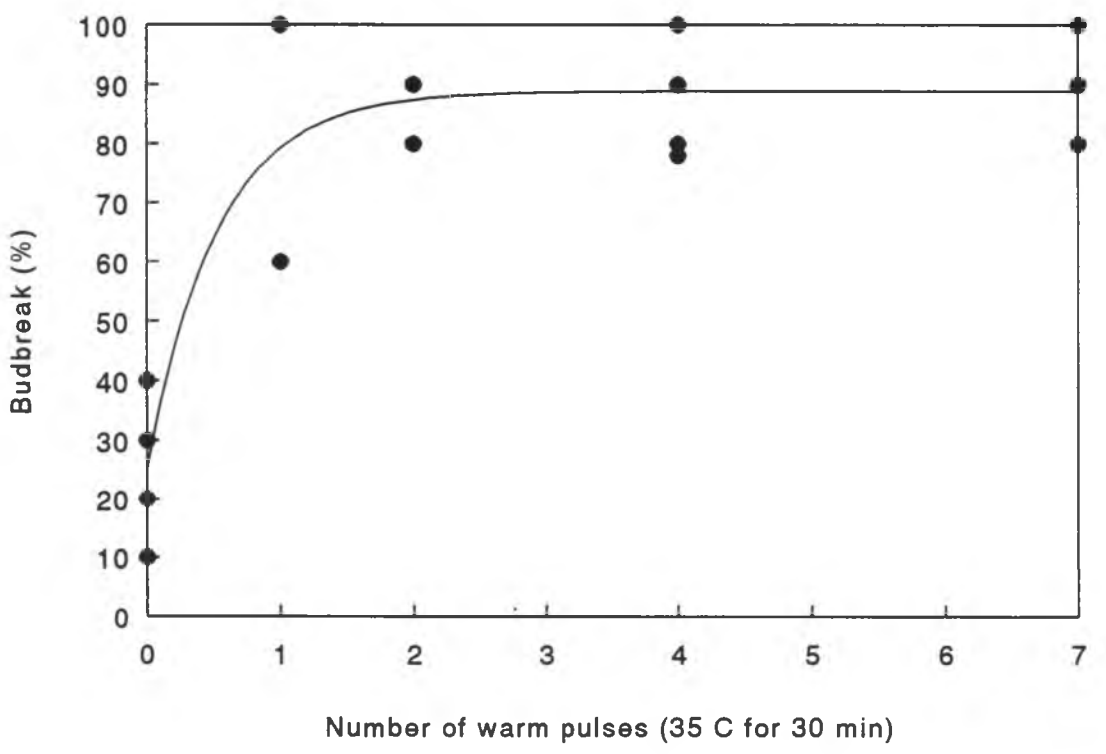

Fig. 3.1. Budbreak of purple nutsedge tubers in response to an increasing number of thermal pulses of 35C for 30 min, and 20C for 23.5 h in a shift cycle; after the thermal pulse(s), tubers were returned to constant 20C. The value for budbreak at constant 20C is at zero pulse. Observations with the same value are hidden from view. The fitted model is $Y = 88.90 - 63.84 * \exp(-X/0.53)$; $r^2 = 0.809$. $n = 4$.

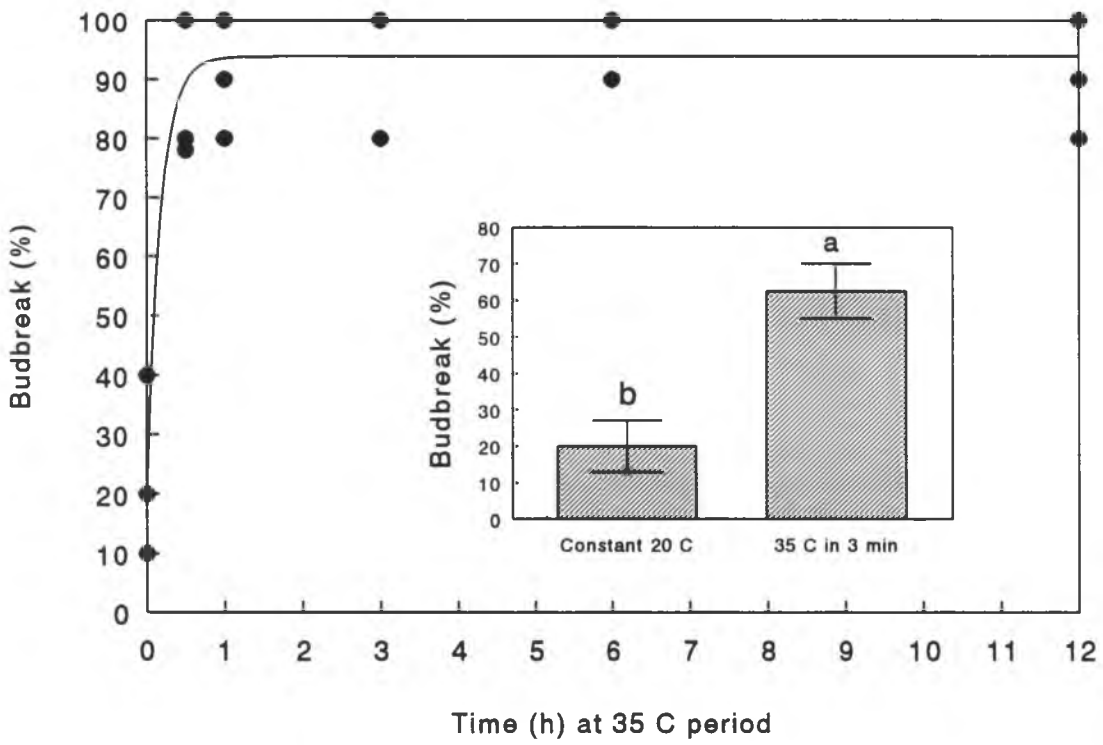

Fig. 3.2. Effect of duration at 35C with a single thermal fluctuation on tuber budbreak; after the single temperature shift, tubers were returned to constant 20C. The value for budbreak at constant 20C is at 0 h at 35C period. Observations with the same value are hidden from view. The fitted model is Y = 93.85 - 73.84 * exp (-X/0.18); $r^2 = 0.898$. n = 4. Budbreak enhancement by a 3-min pulse of 35C is shown in the bar graph insert. Bars with different letters differ significantly by Duncan's multiple range test, P=0.05.

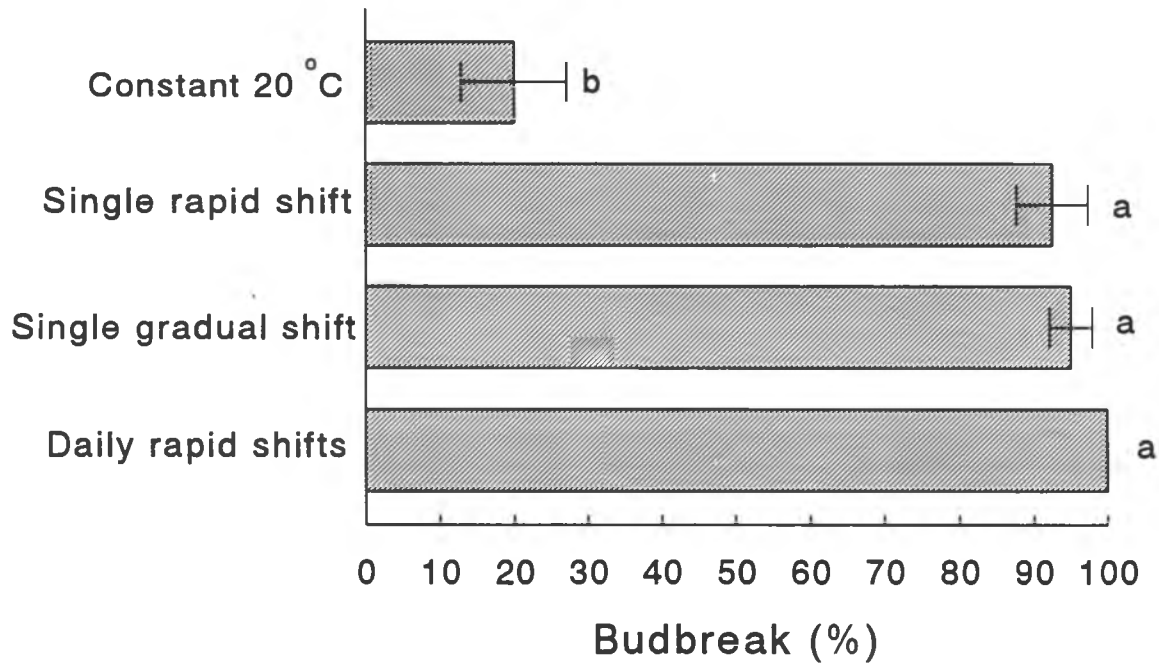

Fig. 3.3. Comparison of tuber budbreak in response to two different rates of temperature shifts. A sand buffer within an incubator provided a gradual shift in temperature, and a rapid temperature shift was generated in an incubator. Both shifts have the same maximum temperature and the same accumulative heat unit (Deg > 20C x Time (min)). The daily alternating temperature (daily rapid shifts), had the same rate of temperature increase as the single rapid shift. Horizontal bars represent SE. Bars with different letters differ significantly by Duncan's multiple range test, P=0.05. n = 4.

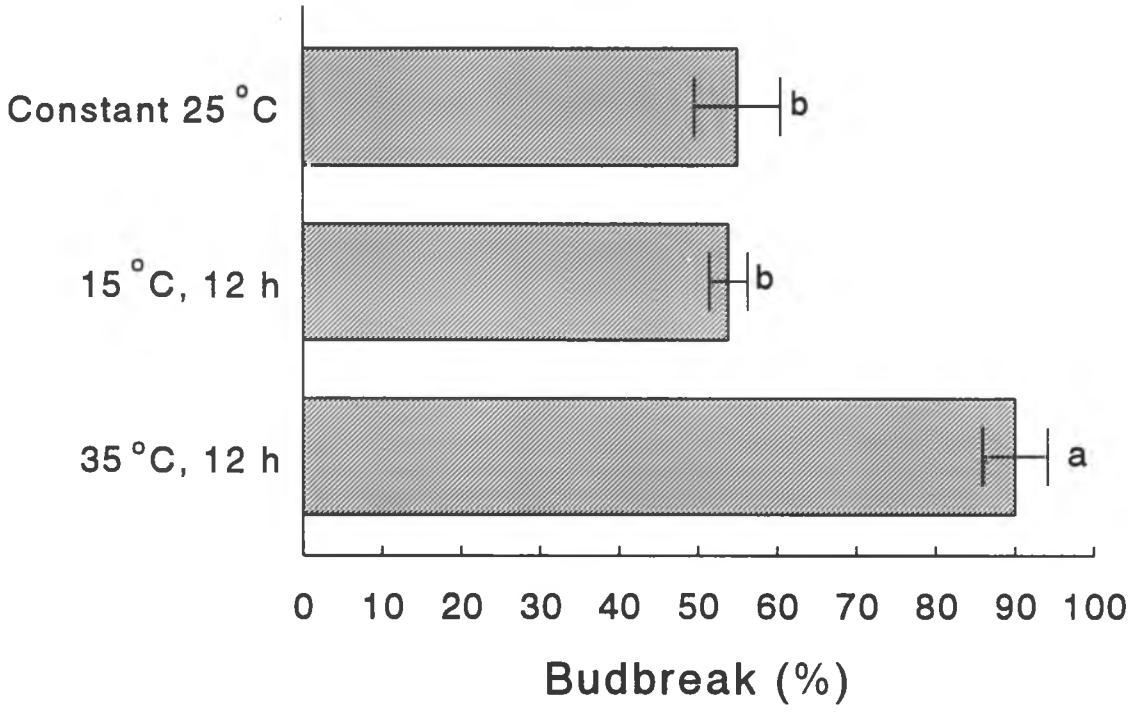

Fig. 3.4. Evaluation of upper and lower temperatures on the budbreak of purple nutsedge tubers. Constant 25C was used for pre-incubation and selection of dormant tubers and for the base temperature in the both temperature shifts. The number of sprouted tubers was recorded 5 d after the temperature shifts. In the cold shift, temperature declined from 25C to 15C for 12 h, while in the warm shift, temperature increased from 25C to 35C for 12h. Horizontal bars represent SE. Bars with different letters differ significantly by Duncan's multiple range test, P=0.05. n = 4.

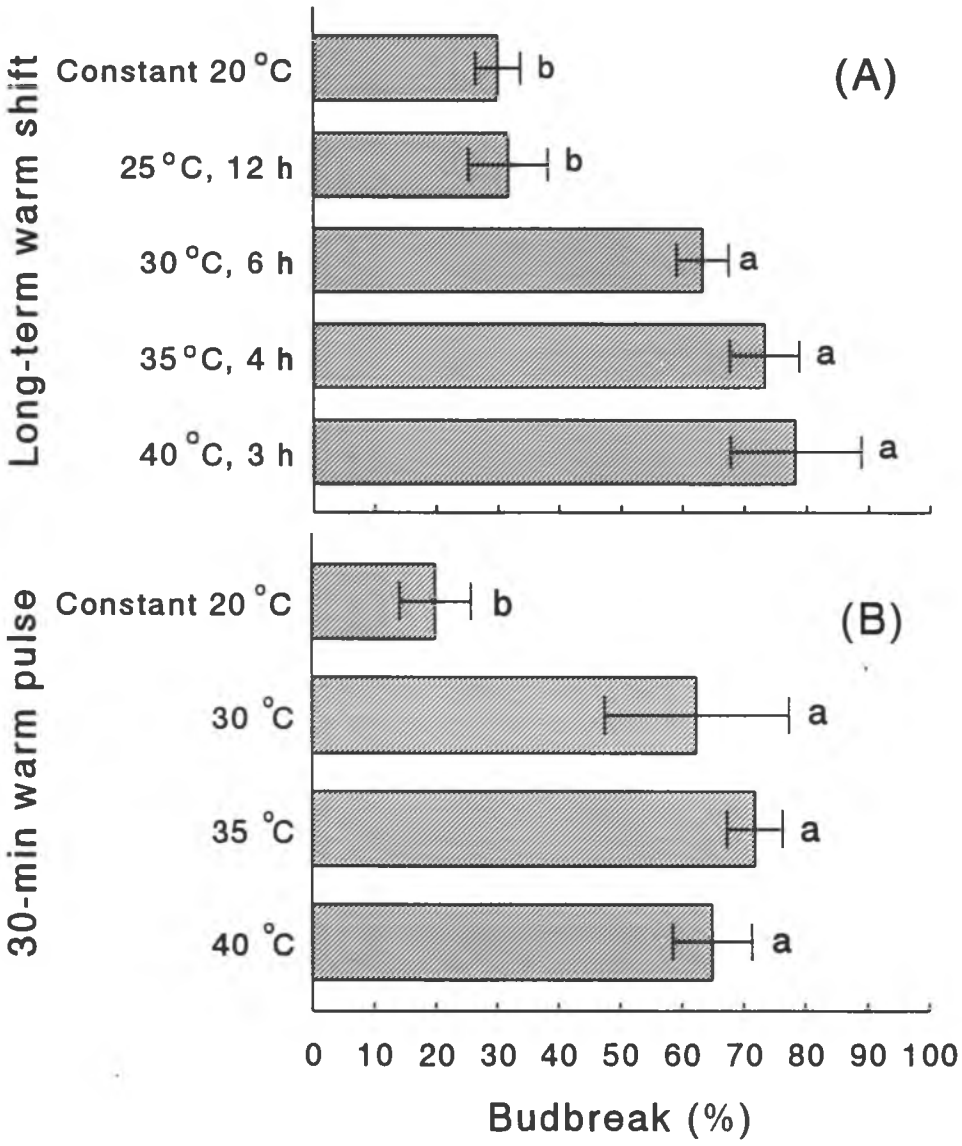

Fig. 3.5. Effect of magnitude of a single temperature shift on budbreak. Each thermal fluctuation has the same heat unit (Deg > 20C x Time (min), 3600C·min) in the long-term warm treatments (a). In the short-term warm pulses, tubers were exposed to a single 30-min elevated temperature shift (b). Horizontal bars represent SE. Bars with different letters differ significantly by Duncan's multiple range test, P=0.05. n = 4.

CHAPTER IV

INVOLVEMENT OF INTRACELLULAR CA^{2+} IN HEAT PULSE-INDUCIBLE BUDBREAK OF PURPLE NUTSEDGE TUBERS

Introduction

There are a few studies on the effect of heat pulse on plant morphogenesis. A single heat pulse treatment induces seed germination (Isikawa, 1975; Vicente et al., 1968; Taylorson and Hendricks, 1972a; Taylorson and Dinola, 1989), and budbreak (Halevy et al., 1964; Sun, Chapter III). However, little is known about how plants sense the changes in temperature and how this information is translated into a physiological response.

Calcium has been suggested as a universal messenger in transducing various environmental and hormonal stimuli, such as wind, touch, wounding, gravity, light , cold shock, elicitor, mechanical, hypoxia, redox, salinity, cytokinins, GA, ABA and IAA; and in coupling many stimulus-responses including tip growth in root hairs and pollen tubes, germination, trophic movements, stomatal closure, and cytoplasmic streaming in plants (see reviews by Hepler and Wayne, 1985; Poovaiah and Reddy, 1993; Subbaiah et al., 1994; Bush, 1995). In animal cells, heat profoundly alters cellular Ca^{2+} homeostasis, inducing Ca^{2+} influx into the cytoplasm from both internal stores and the extracellular medium (Calderwood et al., 1988; Drummond et al., 1986). In plants, heat pulse failed to alter cytosolic Ca^{2+} in transgenic tobacco seedlings (Knight et al., 1991). However, increasing evidence for calcium involvement in the heat responses (Vidal et al., 1993; Braam, 1992; Ling et al., 1994; Parlati et al., 1995; Miernyk et al., 1992; Harrington et al., 1990; Harrington et al., 1994; Lu and Harrington, 1994), suggests that Ca^{2+} may act as a messenger in thermal signal transduction pathway in plant system.

Plasma membrane Ca^{2+} channels have been suggested to be the principal routes of Ca^{2+} entry in animal and plant cells (Hofmann et al., 1994; Bush, 1995). Some of the earliest evidence for Ca^{2+} involvement in regulation of metabolism and in signal transduction in plant single cells and intact plants was obtained through the use of pharmacological agents that block the opening of animal cell voltage-dependent Ca^{2+} channels (Saunders and Hepler, 1983; Reddy et al., 1988; Knight et al., 1992; Schiefelbein et al., 1992, Subbaiah et al., 1994). Taking advantage of the ability of verapamil (phenylalkamine) to bind to membrane proteins, components of putative Ca^{2+} channels were isolated (Graziana et al., 1988; Harvey et al., 1989; Thuleau et al., 1993), and a 75-kDa protein that has Ca^{2+} channel activity in the artificial lipid bilayer has been identified from carrot membranes (Thuleau et al., 1993). Molecular mechanisms of three major classes of organic Ca^{2+} antagonists, represented by verapamil, diltiazem and nifedipine, have been well characterized in animal cells (Hofmann et al., 1994). However much less information on these compounds is available in plant cells (Pragnell et al., 1994; Muta et al., 1994). Owing to unique plant structures, such as cell wall, the agents may have difficulty in reaching the target channel proteins in plant cells; concentrations used in single plant cells or multiple cellular intact plants are 100 to 1,000-fold higher than in animal cells (Lee and Tsien; 1983; Takagi and Nagai, 1988; Wilkinson and Duncan, 1993; Jackson and hall, 1993). Potential side effects arising from such high concentrations, and unknown specificity of the pharmacological agents in plants are important considerations when using these chemicals.

In this initial investigation of the role of Ca^{2+} in the heat pulse-stimulated budbreak of purple nutsedge, The effects of a wide range of compounds that interfere with cytosolic Ca^{2+} levels and others that may be indicative of Ca^{2+} participation in the signal tranduction pathway were examined. These compounds include Ca^{2+} channel blockers

(verapamil, diltiazem, nifedipine, and La^{3+}), Ca^{2+} competitor (Cd^{2+}), Ca^{2+} chelator (EGTA), and Ca^{2+} ionophore (ionomycin).

Materials and Methods

Excised bud. The excised bud system of Teo et al. (1974) was followed with several modifications. The growth and selection of purple nutsedge tubers were the same as described in Chapter III. All lateral buds from unsprouted tubers were used in this study. A piece of tuber tissue 2 to 3 mm in thickness, containing a bud was peeled, and cut into a 3 mm disk with a cork borer. The excised buds were immediately immersed and incubated for 30 min in a medium containing 20 mM MES, and 0.5% Polyvinylpolyrrolidone (PVP), adjusted to pH 5.6 with 1 M NaOH. The excised buds were disinfected in a 0.3% solution of Captan (3a,4,7,7a-Tetrahydro-z-[(trichloromethyl)thio]-1H-isoindole-1,3 (2H) -dione) with shaking for 20 min. The explant disks were washed five to six times each in sterile deionized water and then placed on filter paper to absorb extra surface water. The temperature for the process was controlled at 20 ± 2 C prior to treatments. Relative humidity (RH) in all incubators or ovens was 60% to 70%.

Bioassay. For each treatment, ten excised buds were soaked for 2 h in a fresh PVP-free MES solution with or without the effectors tested at the indicated concentrations. A bud in which the scale leaf (similar to coleoptile) emerged out of the bud covering with a microscopic observation was counted as a sprouted bud. After 7 d, the number of sprouted buds was recorded, and expressed as percent budbreak over viable excised buds in each Petri dish. The viability of non-break excised buds was estimated by straining the half-split buds with TTC as described in Charpt III (Fig. 4.1). In most cases, their viability was ca. 100%.

Ca^{2+} antagonist experiments. The chemical-treated buds were placed in Petri dishes (55 mm in diameter) on two pieces of Whatman No.1 filter paper containing 1.5 ml of the MES buffer solution. One-half of the excised buds were placed in a 35C incubator for 1 h. The time to reach 35C was approximately 30 min, and this time was included in the heating times reported. After heat treatments, the buds were transferred into a 20C incubator for 30-min equilibration and then washed with deionized water for five to six times to remove the residues of the effectors before reincubated at 20C in the incubation medium. The other half were incubated for 1.5 h at 20C without the heat treatment as a control for each effector.

EGTA experiments. After heat pulse treatments in the presence of EGTA, the excised buds were washed with 10 mM $CaCl_2$ four to five times, then soaked in 1 mM $CaCl_2$ for 30 min. To evaluate the poststress recovery of the EGTA treated buds, two supplementary treatments were used in the experiment. One was to expose excised buds for 1 h at 35C that had been previously incubated at 20C with 40 mM EGTA, and washed with 10 and 1 mM $CaCl_2$ respectively. The other was to incubate the buds in 40 μM ionomycin for 2 h following the 35C heat pulse in the presence of 40 mM EGTA, and the washing steps as described above.

Ionophore experiments. Excised buds were submerged in various concentrations of ionomycin containing 1 mM Ca^{2+} for 2 h, and then immediately washed with deionized water and incubated in the incubation medium at 20C. To examine whether Ca^{2+} was required for budbreak, and whether treatments of Ca^{2+} and heat pulse effected budbreak in a similar process, excised buds were treated with 40 μM ionomycin combined with 20 mM EGTA for 2 h, and with 40 μM ionomycin for 2 h, followed by a 35C heat pulse for 1 h in the absence of ionomycin.

Chemicals. All chemicals used in the study were analytical or ultrapure grade, and purchased from Sigma Chemical Co. or Calbiochem. Nifedipine was dissolved in acetone

and mixed with Tween 20 (final concentrations of acetone and Tween 20 did not exceed 4% and 0.1%, respectively); the same solvent and detergent concentrations were used in the control for nifedipine. Ionomycin was initially dissolved in DMSO, and then diluted in the 20 mM HEPES buffer containing 1 mM Ca^{2+}, pH 7.6 (final concentration of DMSO did not exceed 5% (v/v) in the solution). The excised buds for the ionomycin control were treated with the same solution without ionomycin. Other Ca^{2+} antagonists, EGTA were dissolved in a MES (20 mM. pH 5.6) buffered solution. Light-sensitive verapamil and nifedipine were processed in darkness or under safe light.

Results

Effect of calcium antagonists on heat pulse-stimulated budbreak. Single excised buds exposed to an elevated temperature of 35C for 1 h (a heat pulse) stimulated budbreak (Fig. 4.2); their budbreak significantly increased over 20C controls (Table 4.1, Fig. 4.3 and 4.4). Preliminary experiments showed that verapamil, diltiazem, and nifedipine decreased bud emergence of heat pulse-treated excised buds in a concentration-dependent manner from 1 to 1,000 μM. At 1 mM, budbreak was higher than the control without the heat pulse, suggesting that 1 mM of the effectors tested was not sufficient to completely reverse the heat pulse-stimulated budbreak, and there was no toxicity of these effectors at the 1 mM level. To maximize the inhibition of antagonists, concentration ranges of 1 and 10 mM were used in the comparison of bud emergence of Ca^{2+} antagonist-treated buds at 20C versus heat pulse (Table 4.1). Of the chemicals tested, verapamil, diltiazem, and nifedipine at 1 mM level had no adverse effect on budbreak at constant 20C, and verapamil, diltiazem, and lanthanum slightly reduced budbreak of heat pulse-treated buds. Ten mM verapamil and diltiazem increased the inhibition, and also impaired budbreak at constant 20C. Ten mM of nifedipine was not included in the experiment since nifedipine

at such high concentration failed to be dissolved completely. One and ten mM of cadmium and ten mM of lanthanum strongly inhibited budbreak with heat pulse treatment, and also had toxic effects on budbreak at constant 20C (Table 4.1). This made it difficult to distinguish the specific effects of lanthanum and cadmium.

EGTA prevention of heat pulse effect on budbreak. The effect of EGTA on budbreak was concentration-dependent at the range from 0 to 20 mM. Increasing concentration of EGTA from 0 to 10 mM slightly reduced budbreak of heat pulse-treated buds (Fig. 4.3), but from 10 to 20 mM, budbreak decreased markedly; increasing EGTA to 40 mM caused no significant decrease in budbreak compared to 20 mM EGTA (Fig. 4.3). Removing the EGTA from around the bud tissues and replacing with Ca^{2+} following the 2-h EGTA stress (20 and 40 mM EGTA), percent budbreak was the same as the control without EGTA at constant 20C (data no shown). In addition, excised buds that were previously stressed by EGTA responded to heat pulse in the absence of EGTA; 40 μM ionomycin completely reversed EGTA inhibition of the heat-shock-stimulated budbreak (Fig. 4.3, insert). Thus, high concentrations of EGTA did not affect the recovery of the stressed excised buds and the effect of EGTA on budbreak was reversible. It was estimated that 2-h exposure of 20 and 40 mM EGTA reversed approximately 90% of the heat pulse-stimulated budbreak.

Substitution of calcium ionophore for the heat pulse effect. At constant 20C, the emergence of excised purple nutsedge buds linearly increased with an increase in concentration of ionomycin over the range from 0 to 40 μM (Fig. 4.4). The budbreak with 40 μM was equivalent to the heat pulse treatment of 35C for 1 h. When excised buds were preincubated with 20 mM EGTA for 2 h to deplete apoplastic Ca^{2+}, they failed to undergo budbreak even in the presence of ionomycin, which confirmed that Ca^{2+} was required for the budbreak. Again, a single heat pulse following a 2-h incubation with 40 μM ionomycin resulted in similar budbreak to those treated only with a heat pulse

treatment or only with ionomycin (Fig. 4.3, insert). Taken together, calcium ionomycin substituted for the heat pulse stimulation of budbreak.

Discussion

A positive correlation between an increase in the intracellular Ca^{2+} and the heat pulse-inducible purple nutsedge budbreak has been suggested through facilitating or artificially blocking the influx of cytosolic Ca^{2+} in the excised bud system. The concentrations of Ca^{2+} effectors tested in these experiments had been used in other multiple cellular plant systems (Wilkinson and Duncan, 1993; Jackson and Hall, 1993). However such high concentrations of the agents may cause various side-effects in different plant materials (Hepler and Wayne, 1985), and interfere with interpretations of experimental data. Because of this possibility, appropriate experimental controls were used. In the case of excised purple nutsedge buds, concentrations higher than 1 mM of $LaCl_3$ and $CdCl_2$, and 10 mM of verapamil and diltiazem caused considerable budbreak inhibition. On the other hand, 1 mM of verapamil and diltiazem slightly reduced budbreak; 20 to 40 mM of EGTA completely inhibited emergence of excised buds in the presence of heat pulse. The same concentration of these inhibitors had no adverse effects on budbreak in the 20C controls. Thus, the toxicity and antagonistic effects were caused by the inhibitors. Budbreak can be induced by exposure to Ca^{2+} ionophore to the magnitude attained by heat pulse. Therefore, it is reasonable to suggest that Ca^{2+} acts as a second messenger for the heat stimulus.

The excised bud system allowed us to obtain larger experimental sample numbers, and permit easier application of Ca^{2+} effectors. However, the budbreak of excised buds exposed to a single 35C heat pulse was less than 70%. It is difficult to compare the heat pulse effect of the excised bud system with the intact tuber ($\geq$3 lateral buds per tuber)

system used in Chapter III, where the tuber was counted as sprouted when one or more buds emerged. It was estimated that the average budbreak of all lateral buds in an intact tuber at constant 20C and after an exposure to a single heat pulse (35C, 30 min) was less than 10% and ca. 40% respectively, while budbreak without or with the heat pulse treatment in the excised bud system usually was ca. 40% or 65% respectively. Thus, emergence of excised buds without the heat pulse is higher than expected. This stimulation may be caused by wounding (Barckhausen, 1978).

Much effort was made to improve the sensitivity of the excised bud system to heat pulse. Tissue browning is suggested as an indicator of oxidized phenolic compounds, released for the cut ends of the explants by polyphenoloxidases (Mayer and Harel, 1979), peroxidases (Vaughn and Duke, 1984) or air (Robinson, 1983). Release of these compounds might be inhibitory to bud emergence (Teo et al., 1974), and interfere with the heat pulse response of excised buds. Thiourea is an effective phenoloxidase inhibitor (Driessche et al., 1984). Five mM thiourea reduced budbreak both with and without heat pulse (data not shown). However it is not suitable for use in a culture medium because thiourea is a copper-chelating agent (Driessche et al., 1984); long-term incubation with this agent may affect other copper-dependent enzymes such as ascorbic acid oxidase, tyrosinase, monoamine oxidase, uricase, and cytochrome oxidase, and thereby interfere with the heat pulse response. PVP was reported to improve stability of enzymes by removing phenolic impurities, and prevent tissue browning (Gupta et al., 1980). PVP (0.5%) reduced budbreak by10% at 20C over the incubation medium without PVP, but only slightly increased budbreak with heat pulse treatments (data not shown). Since PVP is a hydrophobic compound, it may chelate other hydrophobic Ca^{2+} effectors tested. Therefore, PVP was used only in the MES medium without other organic compounds.

Inorganic low pH buffer, MES was used, instead of phosphorus buffer (Teo, et al., 1974) because phosphorus can precipitate some effectors such as La^{3+} as well as intracellular and extracellular Ca^{2+}, which might interfere with the heat pulse response.

Captan appeared to be much more effective at controlling fungi in purple nutsedge tubers than disinfectants such as ethanol (70%), and sodium hypochlorite (1.5%), resulting in increasing the heat pulse response of excised buds by 10% to 15%, and better experimental reproducibility (data not shown). In addition, maintaining incubator RH at 70% facilitated the control of fungi of purple nutsedge excised buds.

Preliminary experiments revealed that 1 h of bud incubation at 35C provided highest budbreak over other tested times from 0.5 to 4 h (data not shown). Taken together, the improved excised bud system was a useful method to assess the effects of any chemical reagent on the stimulus-inducible budbreak. The incomplete induction of purple nutsedge bud emergence suggested that temperature was not the only factor controlling the dormancy release. It was reported that 1 ppm BA resulted in 100% budbreak of excised buds at constant 30C (Teo et al., 1974).

The entry of Ca^{2+} across the plasma membranes or calcium store organelles is believed to be a highly complex system dependent upon multiple cation channels, multiple ATPases, and probably specific anion channels (Wilkinson and Duncan, 1993). Three classes of gated Ca^{2+} channels have been detected in plants; they are voltage-operated, second-messenger-operated or ligand-gated channels, and mechanically operated Ca^{2+} channel. Three organic Ca^{2+} channel blockers, verapamil, diltiazem, and nifedipine inhibit only voltage-operated channels presumably by attaching to a receptor site at, or close to, the plasma membrane surface (Takagi and Nagai, 1988; Bae et al., 1989). The reversal of the heat pulse-inducible purple nutsedge budbreak was partial and varied with the three different Ca^{2+} channel blockers indicating at least three possibilities. The first was that different channels may have different sites for the ion channel blockers as proposed by

Wilkinson and Duncan (1993). The second was that other types of Ca^{2+} channels may serve as Ca^{2+} entry pathway for purple nutsedge cells. Stretch-operated channels (one kind of mechanically operated Ca^{2+} channels) have been proposed to be involved in turgor regulation (Cosgrove and Hedrich, 1991) and temperature response (Ding and Pickard, 1993b). Such stretch-operated channels were specifically inhibited by Gd^{3+} (Yang and Sachs, 1989), and Loadlacquer stretch dye (Jones 1988). The third was that increased cytosolic Ca^{2+} may be derived from intracellular Ca^{2+} stores.

Although the exact source of Ca^{2+} release in purple nutsedge bud cells remains to be determined, the response appears to be dependent on extracellular Ca^{2+} based on several lines of evidence. The effectiveness of plasma membrane Ca^{2+} channel blockers on the heat response indicates the Ca^{2+} might be, at least partially, mobilized through verapamil-, and diltiazem-sensitive trans-plasma membrane Ca^{2+} channels. Since EGTA, as a polycarboxylic compound, effectively sequesters apoplastic Ca^{2+} but does not reach the intracellular stores (McAinsh et al., 1991; Himpens et al., 1992; Tsien, 1992), the almost complete reversal of heat-shock-stimulatory effect on budbreak (Fig. 4.3) suggests that the extracellular Ca^{2+} might be a major source of Ca^{2+} influx.

It should be pointed out, however, that other contributing mechanisms for the heat-induced rise of intracellular Ca^{2+} ($[Ca^{2+}]_i$) cannot be ruled out. In response to heat in animal cells (HA-1 fibroblasts), elevated $[Ca^{2+}]_i$ initially results from release of Ca^{2+} from an internal store, probably located in the endoplasmic reticulum, followed by influx of Ca^{2+} from the extracellular medium correlated with turnover of the phosphoinositides (PI) (Calderwood et al., 1988). Heat pulse induced increases in cellular inositol triphospate (IP_3) occurs within 15 seconds in sugarcane, suggesting the probable presence of a heat target as in the animal system (H.M. Harrington, personal communication). In addition, mitochondria and the vacuole contain large pools of stored Ca^{2+} (Campbell, 1983; Bush, 1995) that could be targets for heat . It is clear that further work is required to determine

the contribution of different Ca^{2+} sources to the heat-induced $[Ca^{2+}]_i$, and the precise role of the heat-induced Ca^{2+} changes in the process of dormancy release of purple nutsedge tubers.

Table 4.1. Effect of Ca^{2+} antagonists on heat pulse-stimulated budbreak of excised purple nutsedge buds.

Antagonist	Concentration (mM)	Budbreak (%)	
		20C[a]	35C Heat pulse
Control		42.5 a	60.0 a
Verapamil	1	40.0 a	47.5 ab
	10	15.0 d	27.5 b
Diltiazem	1	42.5 a	47.5 ab
	10	20.0 cd	32.5 b
$LaCl_3$	1	30.0 abc	40.0 ab
	10	27.5 bcd	27.5 b
$CdCl_2$	1	15.0 d	25.0 b
	10	2.5 e	2.5 c
Control[b]		40.0 a	55.0 a
Nifedipine	1	45.0 a	52.5 a

[a] Means within a column followed by the same letter do not differ at the 0.05 probability level, according to Duncan's multiple range test. n = 4.

[b] Acetone control for nifedipine treatment, which contains 4% acetone and 0.1% Tween 20.

Fig. 4.1. TTC strained non-break excised bud of purple nutsedge.

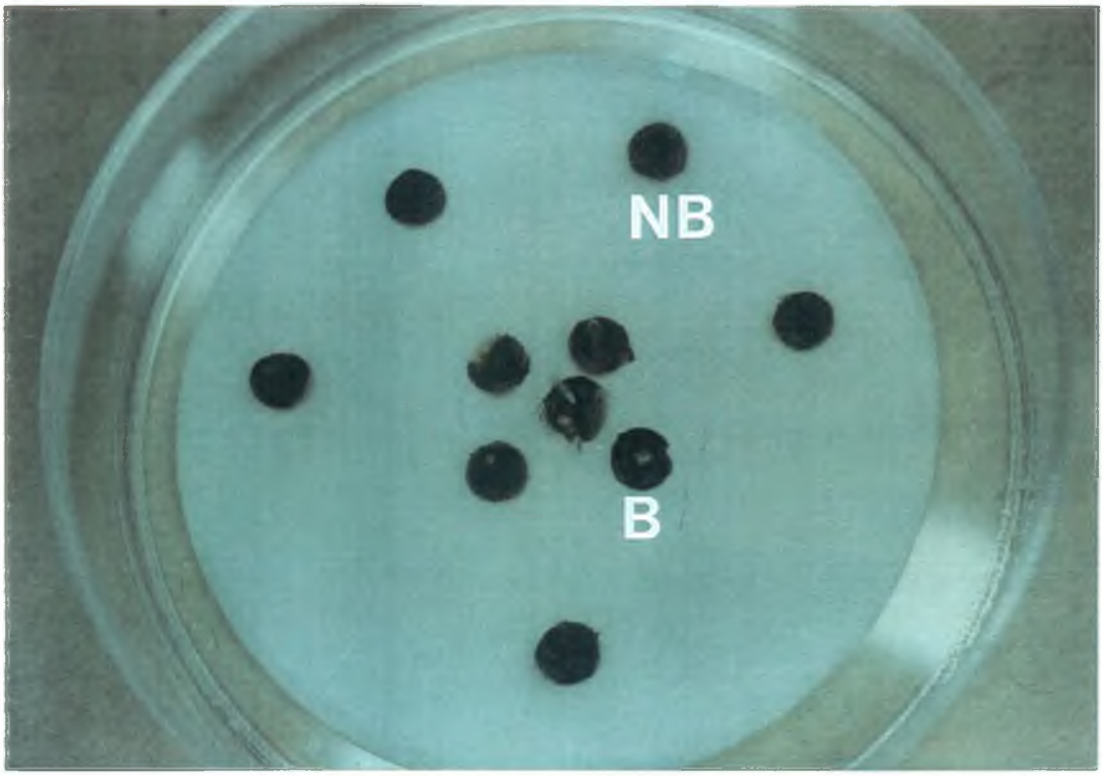

Fig. 4.2. Budbreak (B) and non-break (NB) excised buds of purple nutsedge 7 d after a single heat pulse treatment (35C for 1 h).

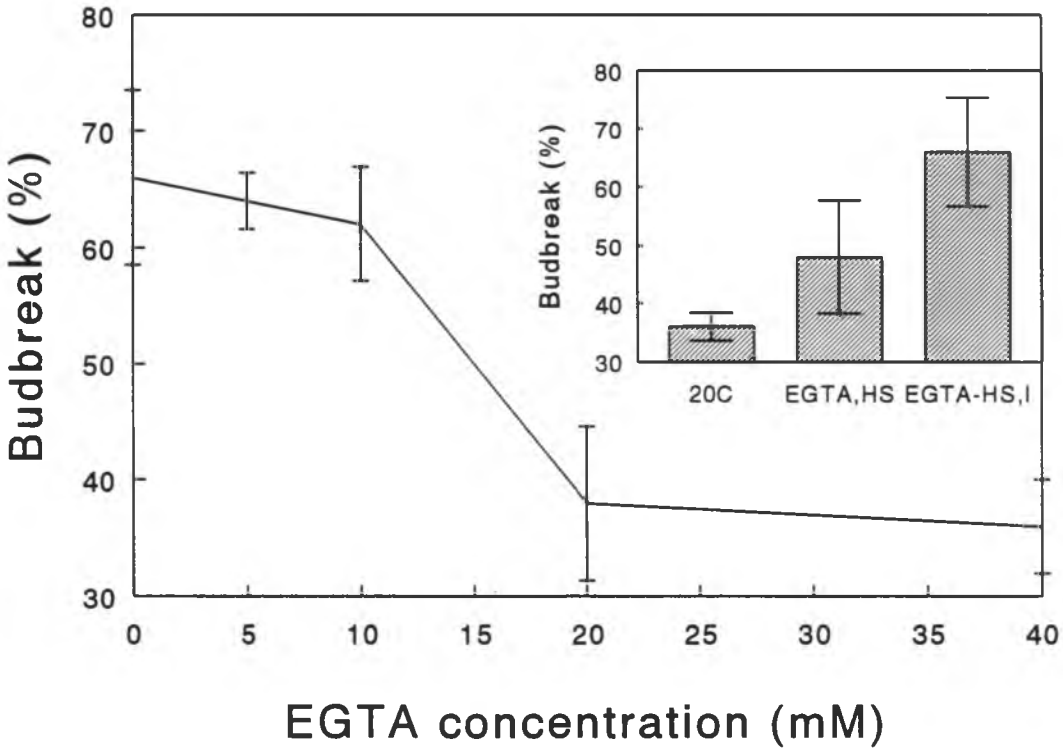

Fig. 4.3. Influence of increasing concentrations of a Ca^{2+} chelator, EGTA on budbreak of heat pulse-treated excised buds. The poststress recovery of excised buds to 40 mM EGTA treatment was examined with treatments including removal of EGTA with 10 and 1 mM $CaCl_2$ and followed by a 1-h 35C heat pulse (EGTA, HS), and removal of EGTA residual after heat pulse treatment in the presence of 40 mM EGTA, and followed by 2-h incubation with 40 µM ionomycin (EGTA-HS, I) (shown in the bar graph insert). Vertical bars represent SE, n = 5.

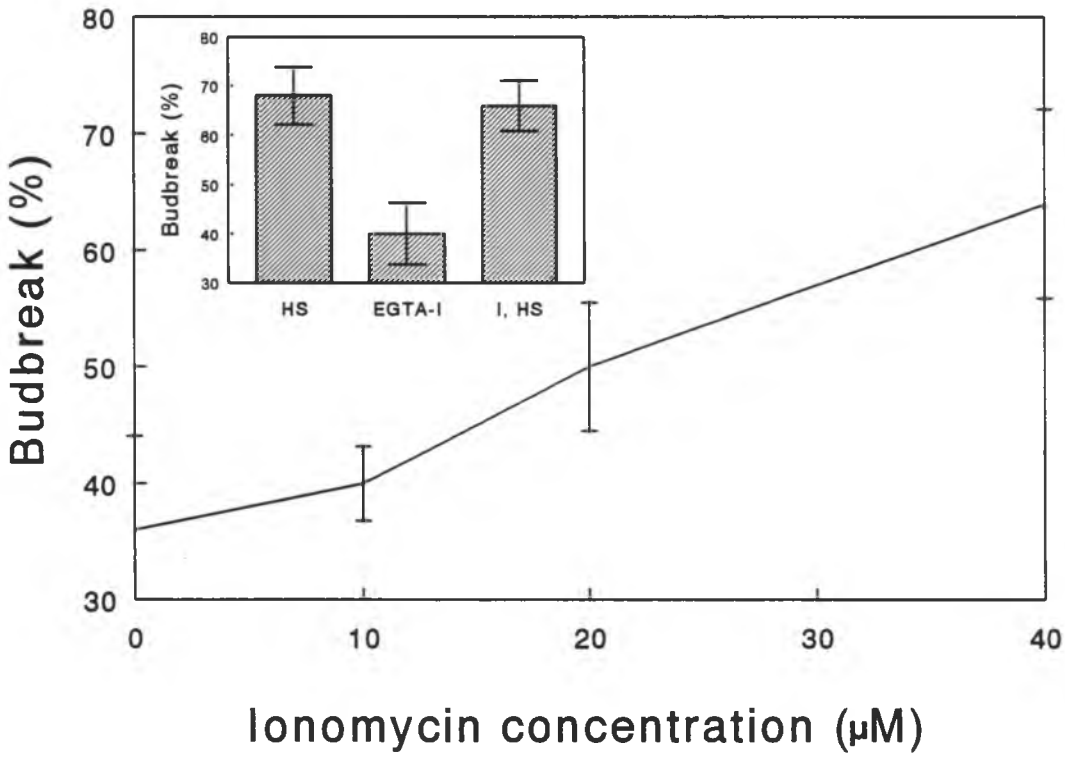

Fig. 4.4. Influence of ionomycin on budbreak of excised purple nutsedge buds at constant 20C. The effect of heat pulse (HS) of 1-h 35C treatment, combination of 40 μM ionomycin and pretreatment with 20 mM EGTA (EGTA-I), and posttreatment with heat pulse (35C for 1 h) (I, HS) is shown in the bar graph insert. Vertical bars represent SE, n = 5.

CHAPTER V

INTRACELLULAR CALCIUM CHANGES IN PURPLE NUTSEDGE CULTURED CELLS IN RESPONSE TO TEMPERATURE FLUCTUATION

Introduction

A crucial criterion for evaluating Ca^{2+} as a messenger in regulating a physiological process is to demonstrate that cytosolic Ca^{2+} changes in response to primary stimulus prior to a physiological reaction (Gilroy et al., 1991; Poovaiah and Reddy, 1993). High temperature-induced cytosolic Ca^{2+} fluxes have been reported in animal systems (Calderwood et al., 1988; Drummond et al., 1986). In plants, however, there has been only indirect evidence for the occurrence or participation of intracellular Ca^{2+} fluxes in response to high temperature. For example, the phosphorylation of threonine residues of heat shock protein Hsp70 is dependent on calcium ions *in vitro* (Vidal et al., 1993). A heat shock-related, calmodulin-dependent glutamate decarboxylase of fava bean roots was produced as a result of external Ca^{2+} (Ling et al., 1994). Heat shock was related to external Ca^{2+} in the expression of the calmodulin-related touch genes of *Arabidopsis* (Braam, 1992). In tobacco cells, a heat shock induces the synthesis of heat shock proteins (hsps) that bind to calmodulin in a Ca^{2+}-dependent manner (Harrington et al., 1990; Lu et al., 1995). I have initiated studies on the role of Ca^{2+} in dormancy release of the purple nutsedge bud by pharmacological methods (Chapter IV). Although the data are highly suggestive of the involvement of $[Ca^{2+}]_i$ in the heat pulse response, direct evidence for intracellular calcium change in intact plant cells needs to be provided to confirm the role of Ca^{2+} in the thermal signal transduction pathway.

There are three major problems for determination of calcium in excised buds: dye loading, autofluorescence, and nonuniformity of physiological stages of unsprouted buds.

While single cells do not reflect the organization or the physiology of intact plant cells, the use of single cells in the study of signal transduction has certain advantages over multiple cellular tissues. First, single cells facilitate imaging studies and quantitative measurement. In addition, single cells are more likely to respond to primary signal sensing and transduction events without the involvement of the slower systemic effects (Subbaiah et al., 1994). In this study, a cell culture system for purple nutsedge was developed, and single cell culture from callus, originally derived from the young shoot tip of purple nutsedge was successfully prepared. To further assess the potential role of Ca^{2+} in triggering the cell mitotic activity, $[Ca^{2+}]_i$ changes were indicated by changes in fluorescence in purple nutsedge-suspension cells during high temperature treatment using laser scanning confocal microscopy (LSCM).

Materials and Methods

Origin of suspension-cultured cells. Purple nutsedge callus cultures were established from tuber disks and shoot tips. Unpeeled tubers were sterilized in 1.5% sodium hypochlorite (28.6% Chlorox) for 20 min. The proximal and distal one-third of tubers were peeled and soaked in 0.1% mercuric chloride for 10 min before tuber removal with a sterile 5 mm cork borer. The plugs of tissue were cut into 20 mm disks; for shoot tips, 1 to 2 mm sections of shoot tips were cut from 5 to 10-mm young tuber sprouts, soaked in 70% ethanol for 1 min, and then replaced in 0.1 % mercuric chloride for 10 min. Sterilized tissues were rinsed five times with sterile deionized water prior to transferring on to the culture media.

The callus was started in a 50 ml Erlenmeyer flask containing 20 ml of MS (Murashige and Skoog, 1962) basal medium plus plant growth regulators (PGR), 3% sucrose, 1% bacto-agar, and pH 5.8 prior to autoclaving. Based on data in potato tuber tissue culture

data (Wang and Hu, 1985; Hagen et al., 1990), six PGR formulations were evaluated for selection of a callus induction medium: 10 μM 2,4-D; 50 μM 2,4-D; 2 μM 2,4-D (2,4-dichlorophenoxyacetic acid), plus 20 μM IAA (indoleacetic acid) and 5 μM KT (kinetin); 1, 5, and 10 μM picloram (4-amino-3,5,6-trichloropicolinic acid). There was one piece of tissue per flask and three flasks per formulation medium tested. After callus initiation a few pieces of aggregates were transferred every 4 weeks into freshly prepared medium containing only 2 μM 2,4-D. For selection of the callus maintenance medium, six other plant growth regulators (PGR) formulations were evaluated: 10 and 50 μM 2,4-D respectively; 2 μM 2,4-D, plus 20 μM IAA; 2 μM 2,4-D, plus 20 μM IAA, and 5 μM KT; 1, and 5 μM picloram respectively. Liquid suspension cultures were established by transferring the callus to the liquid medium containing the same formulation as the maintenance medium, but without agar.

Culture conditions. Cell suspensions were maintained in the dark at 24 to 26C in 125 ml Erlenmeyer flasks containing 30 ml of the liquid medium on a gyratory shaker at 110 rpm and subcultured by transferring 3.5 ml of cell suspension (cell growth towards the end of the exponential growth phase) into 30 ml of the medium every 7 to 10 d. All media were adjusted to pH 5.8 prior to autoclaving. The suspension-cultured cells in their exponential phase of growth (subculture for 7 to 10 d) were used as a source of experiments.

Dye loading. The cells were spun at 1000 rpm for 5 min and resuspended in fresh MS growth medium at pH 5.4 (after autoclave) containing 5 to 20 μM fluo-3 AM, an esterified form of a Ca^{2+}-sensitive fluorescent dye; (diluted with 0.2 μM dimethyl sulfoxide (DMSO)) stock, and 0.02% Pluronic F-127 (according to the protocol of Molecular Probes (Poenie et al., 1986)). Loading was allowed to proceed for 30 to 60 min on the same growth condition (ca. 25C with shaking). To avoid the influence of temperature treatment on dye uptake, extraneous dye was removed by spinning cells at 1,000 rpm for 5 min and resuspended twice in fresh MS medium containing 3 mM Ca^{2+}.

Temperature-shift system. Cells loaded with fluo-3 AM were placed on a microscope slide with a cover slip. The slide was attached to the glass surface of a thermal chamber using vacuum grease. The thermal chamber was modified from a tape box where one surface was replaced with a glass slide, and both sides connected to a recirculating temperature controlled water bath via polyethylene tubing. An immersion circulator (Fisher Scientific) was used to adjust water temperature (± 1C). The chamber temperature could be increased from 20 to 35C in10 to 15 min when the water temperature was set at 40 to 42C. Reducing chamber temperature from 35 to 20C was achieved within 5 min by connecting the circulator to a 20C water bath. The surface temperature of the chamber was monitored with a thermocouple attached to a data logger that could be transferred to a computer and manupilated with the Smartcom II software program.

Fluorescence imaging. The thermal chamber was mounted on the stage of a Nikon Microphot-SA (Nikon Corp., Tokyo, Japan) or Olympus PM-30 epifluorescence microscope. The cells were excited using light from a xenon lamp passed through a 490 ± 10-nm band-pass filter. A Nomarski apparatus was used for some bright-field images. The fluorescence intensity of the images only provided approximate Ca^{2+} content due to unequal thickness of the cells, the reflection of out-of-focus information and the possibility of unequal distribution of the dye in the cell.

After several preliminary experiments, a laser scanning confocal microscope (Bio-Rad MCR-600) equipped with a 25-mW argon-ion laser and dual-channel detection, located at Hawaii Institute of Geophysics on the University of Hawaii at Manoa campus, was used to visualize the intracellular calcium changes of the suspension-cultured cells in response to temperature fluctuations (Fig. 5.5, and Fig. 5.7). The thermal chamber mounted with dye-loaded cells was placed on an inverted Zeiss IM35 microscope plate, and optical sections of 2.5 and 5.0 μm vertical depth were obtained in confocal modes. The cells were

selected using bright-field illumination based on their apparent healthiness (unplasmolyzed and with cytoplasmic streaming) and weak fluorescence that appeared restricted to the cytoplasm. The dye was excited at 488 nm, and the emission was measured at 515 nm. A photomultiplier selected for extremely low dark current was used to detect light at the emission wavelength. The fluorescence intensity was mapped into color pixels with values from 0 to 256. The outer fluorescent region of a whole cell was traced out, and selected for the measurement of cell size and statistical analysis of pixel values. Consequently, results presented from confocal imaging represent an average intensity of Ca^{2+} content over a whole cell. Images were pseudocolor coded to enhance the visualization of the Ca^{2+} signal. The confocal images were made by a Sony video color printer.

Chemical treatments. For detecting the effect of calcium ionophore on the intracellular calcium change, 10 μM ionomycin buffered with 10 mM HEPES, 140 mM KCl, pH 8.5 were slowly added at one end of a cover slip, replacing the culture medium by capillarity with tissue paper.

Results

Callus and suspension-cultured cells. In the first experiment, all 12 explants were contaminated when unpeeled tubers and plugs or shoot tips were treated with 1.5% and 0.5% sodium hypochlorite for 15 to 20 min. Four groups of surface disinfection solutions were used for tubers and plugs in the second experiment: (1) 1.5% and 0.5% sodium hypochlorite for 30 min and 15 min respectively; (2) 70% ethanol for 20 min, followed by 0.5% sodium hypochlorite for 20 min; (3) 0.1% mercuric chloride for 20 min, followed by 0.5% sodium hypochlorite for 10 min; and (4) 1.5% sodium hypochloride for 20 min, and then 0.1% mercury chloride for 10 min. Only tuber disks treated with the group 4 showed no contamination, indicating that the treatment of 0.1% mercuric chloride for 10 min was

more effective than 0.5% sodium hypochlorite for 30 min, and the tuber contained intra-tissue infection. In third experiment, by using the group 4 method for 12 shoot tips and 36 tuber disks, no contamination occurred. Among them, only one shoot tip produced callus on MS medium plus 50 μM 2,4-D. Purple nutsedge callus grew well after 110 d of incubation (Fig. 5.1A). The callus was a light grey to cream color, and some of callus tips contained black pigment (Fig. 5.1B). The MS medium with 2 μM 2,4-D and 5 μM KT provided more black calli. The media with 1 and 5 μM of picloram tended to induce root initiations (data not shown). Callus subcultures grew more rapidly in the medium containing MS with 2 μM 2,4-D alone, and 2 μM 2,4-D plus 20 μM IAA than in the medium with 10 and 50 μM 2,4-D, while the medium with 2 μM 2,4-D produced the callus more friable than with 2 μM 2,4-D plus 20 μM IAA. Thus, 2 μM 2,4-D was considered as an appropriate auxin source and concentration for the maintenance medium.

Suspension cultures were obtained by transfer of friable callus lumps to agitated liquid medium of the same composition as that used for callus growth. The average size of the cells was ca. 35 x 55 m. There were two types of cells in the suspension cultures (Fig. 5.2A). The first usually had one or two nuclei with a large volume of central vacuoles (Fig. 5.1A, bottom), the cytoplasm occurred as a thin layer close to the cell wall and surrounding the vacuole. Some cells had cytoplasmic strands that traversed the vacuole, but each transvacuolar strand was surrounded by the tonoplast (vacuole membrane). In some cells, transvacuolar strands were associated with plastids and nuclei (Fig. 5.2B). The second type of cells had many aggregated granules (Fig. 5.2C). These granules appeared to be starch grains or the oil storage elaioplasts. For the convenience and uniformity in visualization, only the first type of cells were used in all the following experiments.

Dye loading and distribution. In general, suspension-cultured cells fluoresced at around 490 nm without incubation with fluo-3 AM at room temperature. This autofluorescence

was mainly located in the cell wall, and the intensity was variable among the cell population. Increasing cell temperature to 35C for 30 min did not alter the autofluorescence pattern and intensity in the cell wall and in the intracellular region. Cells loaded with 5 μM fluo-3 AM for 30 min showed weak fluorescence in the periphery of the cell, which could be the cell wall or cytoplasmic region (Fig. 5.3B). A marked increase of fluorescence in intracellular cytoplasm was observed after incubation with 10 μM ionomycin for 15 min, and no discernible signal emanated from the vacuole (Fig. 5.3C, cell a, and b), which indicated that the dye fluo-3 AM was absorbed into the cell and cleaved by endogeonous cytosolic hydrolases. Adding 1mM $MnSO_4$ to the cell rapidly reduced the cytoplasmic fluorescence close to the resting level, but the fluorescence on the nuclei and transvacuolar strands remained 10 min after the treatment (Fig. 5.3D, cell c). Finally, After adding 1% Triton X-100, the remaining intracellular fluorescence disappeared in 10 min due to the release of intracellular dye (data not shown). Cells shown in Fig. 5.5 and Fig. 5.7 were loaded with 10 and 20 μM fluo-3 AM for 1 h respectively. Thin optical sections (2.5 and 5 μm) with LSCM showed that before heat treatments, the fluorescent calcium signal was mainly located in cytoplasm (Fig. 5.5A, cell a, c, and d, and Fig. 5.7A), and transvacuolar strands (Fig. 5.5A, cell b), and there was almost no signal inside the vacuole (Fig. 5.5A). These data indicated that 5 to 20 μM fluo-3 AM was loaded into the purple nutsedge cells, the dye was mainly distributed in the cytopasm and transvacuolar strands, and sensitive to Ca^{2+}.

High temperature-inducible changes of $[Ca^{2+}]_i$ in purple nutsedge cells. An intracellular calcium signal response to elevated temperature was visualized with fluorescence microscopy. The group of cells were not plasmolyzed. Cells loaded with 5 μM fluo-3 AM were incubated in the dye-free medium for 10 min at room temperature 24C, and did not show an increase from the resting levels of $[Ca^{2+}]_i$ (Fig. 5.4A, cells a and b; the fluorescence image before dye loading was not shown due to the change of cell

location). With increasing temperature from 24 to 35C in 10 min, fluorescence initially appeared in a very narrow band of peripheral cytoplasm adjacent to the plasma membrane (or cell wall), then into interior regions. Following continual exposure to 35C for another 20 min, the fluorescence in the cytoplasm became brighter, and the peripheral band expanded (Fig. 5.4B, cells a and b); the fluorescent region of transvacuolar cytoplasmic strands expanded and small granule particles appeared (Fig. 5.4B, cells a and c). Another unique feature was that the nucleus surrounded by amylopasts, and transvacuolar cytoplasmic strands holding the nucleus in a central position showed bright fluorescence (Fig. 5.4B, cell c), as identified in the bright-field image of Fig. 5.4C,.

LSCM was used to visualize the $[Ca^{2+}]_i$ changes in response to high temperature signal more precisely. The initial free Ca^{2+} was mainly located in the cytoplasm of cells a, b, c, and d, and the intravacuolar cytoplasmic strand region in cell b (Fig. 5.5A). When the cell temperature was increased from 24C to 35C in 10 min, fluorescent signal in the cytoplasm decreased in all cells, while one more fluorescent strand appeared in cell b (Fig. 5.5B2). After incubation at 35C for 30 min, the fluorescence intensity in both peripheral cytoplasm and the interior regions surrounding the cytoplasmic strands increased (Fig. 5.5B3), and another fluorescent strand was discernible in cell b, and continued increasing up to 60 min in the entire cell (Fig. 5.5B4). Unlike cell b, the fluorescence in the region of transvacuolar cytoplasmic strands in cell a was more diffuse than concentrated in the strand region, and was similar to cell a in Fig. 5.4. These qualitative observations matched the fluorescence intensity analysis data. In the first 10 min, total pixel values in entire cells a, b, c, and d decreased 24.0%, 15.6%, 28.0%, and 40.1% respectively, compared with controls at the resting time. Thereafter, they steadily increased, and eventually reached 141%, 149%, 147%, and 95% of controls respectively, at 60 min of incubation at 35C. Thus the levels of fluorescence in three of four cells increased more than 40% over the initial value (Fig. 5.6A). Levels of the $[Ca^{2+}]_i$ in the middle plane of cells appeared to

fluctuate in response to heat treatment, but was substantially higher after 60 min of incubation at 35C (Fig. 5.6B). The same pattern, in which the time course of change in cytosolic Ca^{2+} oscillated was also seen in another experiment (Fig. 5.8A). Another obvious change of cells in response to the elevated temperature was cell expansion. In the first 10 min after heat treatment, all observed cells enlarged 7% to 11% over the controls. The maximum size of the cells was 10% to 16% larger than the controls at 24C after incubated at 35C for 40 min (Fig. 5.6B).

Response of $[Ca^{2+}]_i$ and cell size to withdrawal of heat stimulus. Of the selected cells, cells b and c (Fig. 5.7A) were similar to cells a, b, and c shown in Fig. 5.4A, in which the majority of resting Ca^{2+} were distributed in cytoplasm, and their fluorescence intensities were much lower than cell a (Fig. 5.7A). When cells were incubated at 35C for 90 min, the fluorescence signal appeared in the regions along with apparently transvacuolar cytoplasmic strands in cells a, b and c (Fig. 5.7C). Results from fluorescence analysis showed that the total pixel values and cell size increased ca. 20% and 18% respectively over the control at zero time during the first 90-min incubation at 35C. The intracellular fluorescence signal was slightly increased to the maximum level 30 min after temperature was rapidly reduced to 20C; the cell size remained at the same level as before the decrease in temperature (Fig. 5.8A). In addition, the Ca^{2+} signal in the regions of cytoplasmic strands of cells a, b and c apparently intensified as incubation time at 35C increased (Fig. 5.7D, cells a, b, and c).

DISCUSSION

The spatial and temporal properties of changes in Ca^{2+} levels induced by heat stimulus were examined. Increasing the temperature 11 to 15C above normal growing temperature caused the intracellular Ca^{2+} level initially to either decrease or increase within 10 min,

then steadily increase (Fig. 5.5B, Fig. 5.7). When the temperature was reduced from 35 to 20C, the Ca^{2+} level did not decline in the first 30 min, which is contrary to the Ca^{2+} signal change in other plant systems with various stimuli such as cold shock, touch, and fungal elicitors (Knight et al., 1991), ATP (Rizzuto et al., 1992), UV light (Kao et al., 1989), and hypoxia or anoxia (Subbaiah et al., 1994). In addition, irregular oscillations in $[Ca^{2+}]_i$ in a single middle plane of cells was observed in purple nutsedge cultured cells (Fig. 5.8, Fig. 5.6B) although steady-state , apparently modest increases in $[Ca^{2+}]_i$ over entire cells occurred. The data indicated homeostatic mobilization of $[Ca^{2+}]_i$ with time in the cells.

Spatially, Ca^{2+} signal commonly increased in the interior region of the cells although changes of Ca^{2+} level in the peripheral cytoplasm vary in different cells: increased (Fig. 5.4B, cells a, and b), or decreased (Fig. 5.5B, cells a, b, and d; Fig. 5.7, cells a, and c), or redistributed (Fig. 5.7, cell b). The elevation of Ca^{2+} signal in the interior region was usually accompanied with formation of localized strand-like forms. Plant vacuoles contain 1,000-fold more Ca^{2+} than cytosol (Bush, 1993), it could be a site that provided a uniformly strong Ca^{2+} fluorescence signal when fura-2/AM and indo-1/AM were loaded in barley aleurone protoplasts (Bush and Jones, 1987; Bush, and Jones, 1988). The increase in $[Ca^{2+}]_i$ was not uniform across the central part of the cell (Fig. 5.5B cell b; Fig. 5.7, cells a, b and c). It is therefore unlikely that the elevated fluorescence signal was generated from the plant vacuole. The fluo-3 AM dye appeared to be excluded from the vacuole and initially was concentrated around the vacuole in the aleurone protoplasts of barley; later the fluo-3 was evenly redistributed throughout the cytoplasm (Gilroy and Jones, 1992). In maize cells, fluo-3 AM was distributed only in the cytoplasm and the nucleus (Subbaiah et al., 1994). The distribution of fluo-3 AM in purple nutsedge cells was similar to maize cells. However, it does not mean that we can exclude another hypothesis that high temperature increases the dye permeability, allowing the de-esterified fluo-3 to leak or be transported from the cytoplasm into vacuole, and increasing the

This might explain the result from Fig. 5.5B4, where the fluorescence in the entire cell "d" was increased slightly after 60 min of 35 C incubation.

One of the characteristics of dividing vacuolated cells is the presence of numerous transvacuolar cytoplasmic strands or bridges, through which the nucleus migrates to the middle of the cell, followed by cell division (Yeoman and Street, 1973; Barckhausen, 1978). It was observed that stress, e.g., wounding, and gravity induced the formation of cytoplasmic strands, thus promoting cell division (Barckhausen, 1978; Volkmann, et al., 1993). The data presented here may agree with their observations. Transvacuolar cytoplasmic strands were frequently found in growing purple nutsedge suspension cells (Fig. 5.2B). Heat pulse stimulated an increase in the formation of cytoplasmic strands which was coincident with an increase in the cytosolic signal (Fig. 5.4B; Fig. 5.5B; Fig. 5.7). When the cytoplasmic strands form and expand transvacuolarly in response to heat pulse, the cytosolic free Ca^{2+} and the dyes move along cytoplasmic streaming in the cytoplasmic strands. Consequently the fluorescence signal appears in the cytoplamic strand regions.

Studies of vacuolated tissue cells and epidermal cells have established that these cytoplasmic strands are mainly composed of actin- and tubulin-based cytoskeleton networks (Traas et al., 1987; Kakimoto and Shibaoka, 1987 Lloyd and Traas, 1988; Bakhuizen et al., 1985, Flanders et al., 1990; Katsuta et al., 1990). How these strands are generated and extended is so far unclear. It is certain that a critical concentration of actin monomers is required for assembly into actin filaments (Lodish et al., 1995). The role of Ca^{2+} in the cytoplasmic streaming has been intensively investigated (Kamiya, 1981). The cytoskeleton, including actin filaments and microtubules, are involved in cytoplasmic streaming and in organelle motility (Allen and Allen, 1978; Kamiya, 1981; Williamson, 1986). Two regulatory factors in the formation of actin filaments, fragmin (Hasegawa, 1980) and gelsolin (Kubinski and Allen, 1995), have been found in plants. Both of them

are activated in the presence of a concentration of free Ca^{2+} higher than 1 μM, and the main function of these proteins is to bind actin and break or sever the actin polymers into shorter fragments (Kamiya, 1981; Lodish et al., 1995) that might provide a source of actin monomers for the formation of cytoplasmic strands. It is notable that the actin-binding activity of gelsolin is inhibited by PIP_2. The reciprocal regulation of gelsolin proteins by the counteracting influence of two second messengers, Ca^{2+} and PIP_2 may result in the Ca^{2+} oscillation in the cytoplamic streaming. When the Ca^{2+} binds gelsolin, the free Ca^{2+} decrease; when the gelsolin is inhibited by PIP_2, the Ca^{2+} releases into cytosol, and the free Ca^{2+} increases. The hypothesis of the Ca^{2+} homeostasis may fit the data presented. The reduction of overall fluorescence intensity in cytoplasm 10 min after the 35C treatment (Fig. 5.5B2) may be due to the use of a large amount of free Ca^{2+} to produce cytoplasmic strands in the cell. Similarly, Ca^{2+} was retained in purple nutsedge cells after the heat stimulus was withdrawn because the cell continually needs Ca^{2+} in the cytoplasmic streaming for the cytoskeleton-associated cell activities.

The role of $[Ca^{2+}]_i$ fluctuation in the control of the actin-myosin interaction is suggested (Wohlfarth-Bottermann, and Gotz von Olenhusen, 1977). The activities of myosin are controlled by Ca^{2+}-dependent calmodulin-like subunits. Also a number of heat shock proteins (hsp), including hsp 70 (Clark and Brown, 1986), 90 (Nishida et al., 1986), and 110 (Nishida et al., 1986), are calmodulin-binding proteins and associate with the cytoskeleton (Calderwood et al., 1988). It is suggested that Ca^{2+} may modulate hsp-cytoskeleton interactions through calmodulin.

The response of individual cells to high temperature was quite variable, as expected. However, cell expansion immediately after exposure to elevated temperature was a common feature of almost every purple nutsedge cultured cell. The heat pulse response of cell enlargement indicated that the initial reaction with temperature may be associated with a change in osmotic potential of the vacuole, leading to increased cell turgor pressure.

Mechanical stretching may trigger calcium channel opening in the plasma-membrane (Pickard and Ding, 1993; Cosgrave and Hedrich, 1991) or tonoplasts (Kluge et al., 1991; Wilkins, 1992), producing a net release of K^+ and Na^+, and entry of Ca^{2+} to signal further regulatory processes (Morris, 1990). It is suggested by the calculations of Sackin (1989) that small-scale swelling of cells (about 10%) would activate stretch-activated channels, bringing Ca^{2+} into the cell (Christensen, 1987; Morris, 1990). Currently, cold temperature-activated mechanosensitive Ca^{2+}-selective channels have been found in the plasmalemma of onion epidermis (Ding and Pickard, 1993). Such mechanical stretching might serve as a key to gate the Ca^{2+} channels of plasma membrane or membrane of intracellular Ca^{2+} stores in purple nutsedge cells since the swelling appears to occur earlier than Ca^{2+} elevation in certain purple nutsedge cells (Fig. 5.5B1 and 2).

In conclusion, this research provides the first direct evidence for the intracellular Ca^{2+} elevation in response to external heat stimulus, and indicate that the major heat-induced changes in Ca^{2+} homeostasis are correlated with changes in the cytoskeleton, such as cytoplasmic strands. Further investigation on the localization of heat pulse-induced $[Ca^{2+}]_i$, regulation of calcium-dependent protein involvement in the activation of microfilaments, and associated cell division and cell elongation, may result in a model by which a single heat pulse triggers dormancy release of purple nutsedge tuber buds.

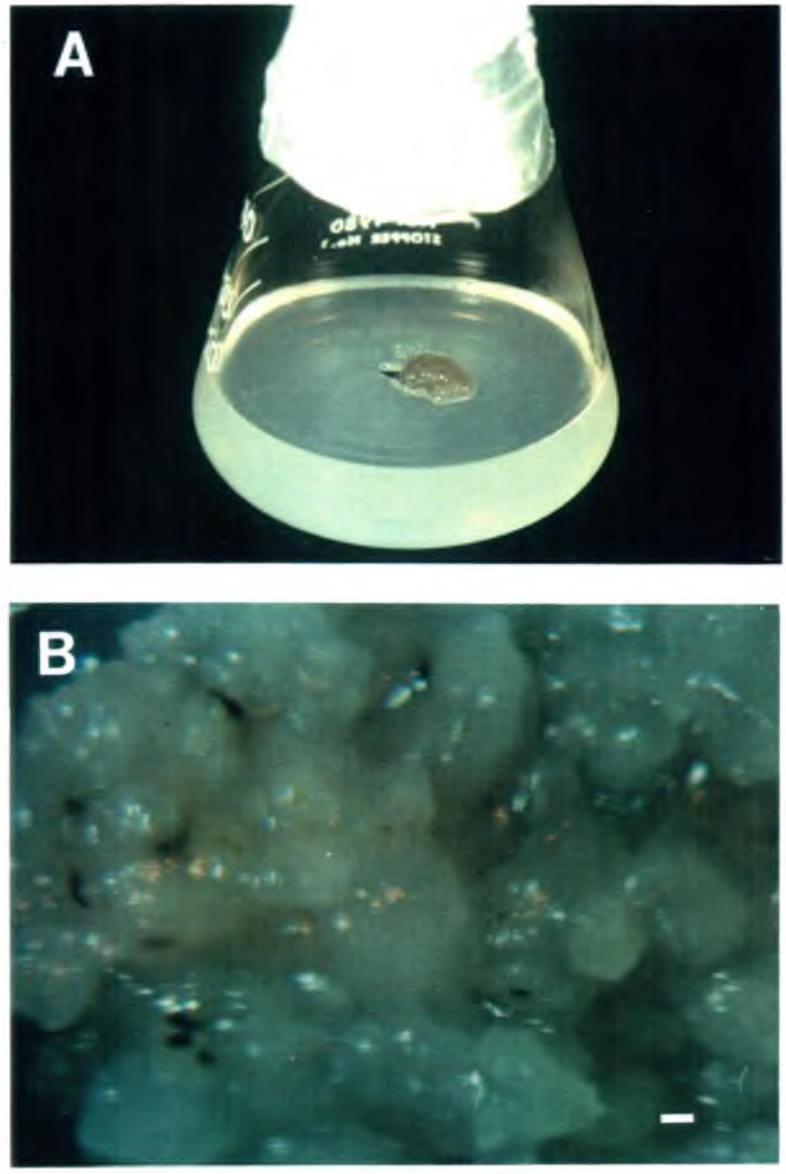

Fig. 5.1. Callus derived from the shoot tip of purple nutsedge. A. Callus was induced in a medium containing MS plus 50 μM 2,4-D. B. Callus was shown at higher magnification. Bar = 500 μm.

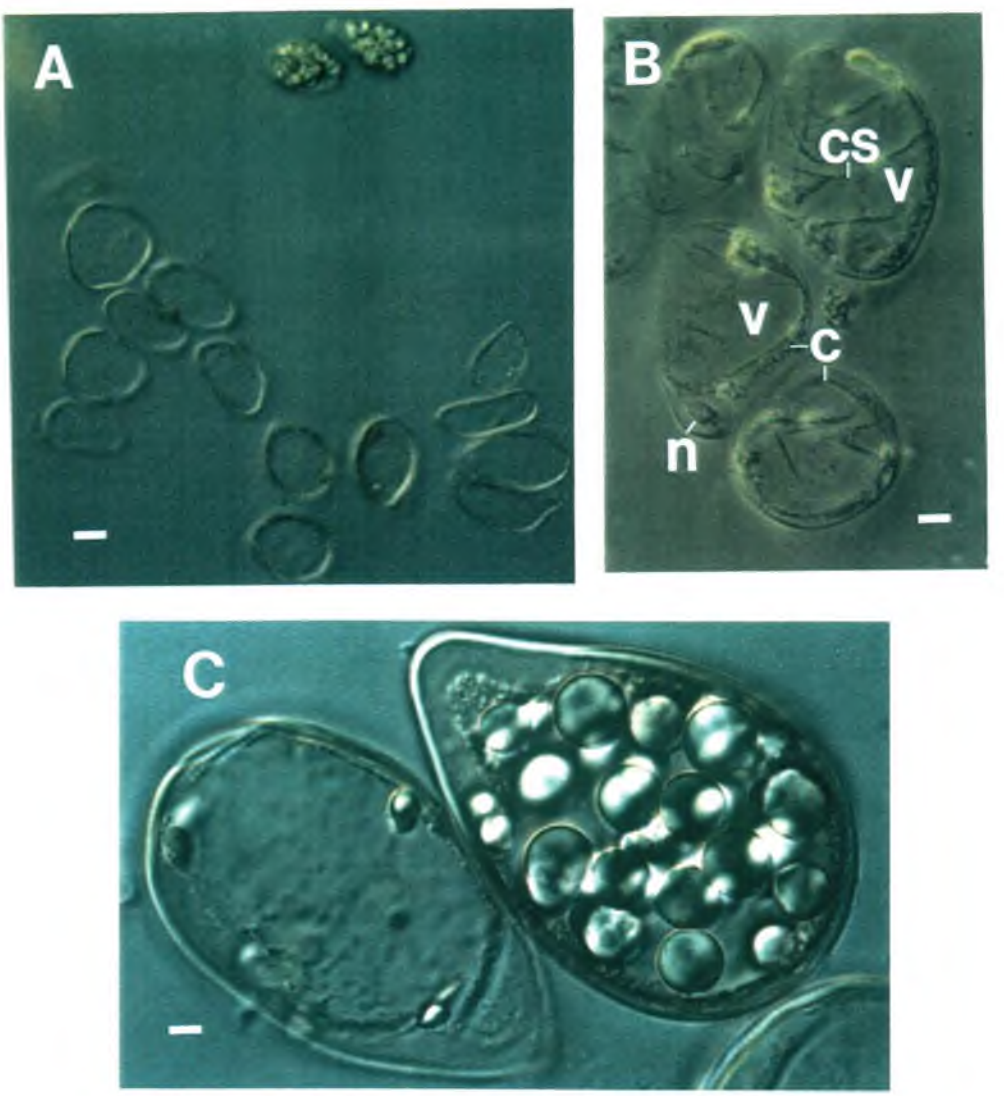

Fig. 5.2. Cells from cell suspension cultures of purple nutsedge. A. Two types of segregated cells in the suspension , photographed with Nomarski interference contrast. Bar = 20 μM. B. Cells consist mainly of nucleus (n), cytoplasm (c), vacuole (v), and cytoplasmic strands (cs). Photographed with Nomarski interference contrast. Bar = 10 μm. C. Cells containing spherical grains. Photographed with Nomarski interference contrast. Bar = 4 μm.

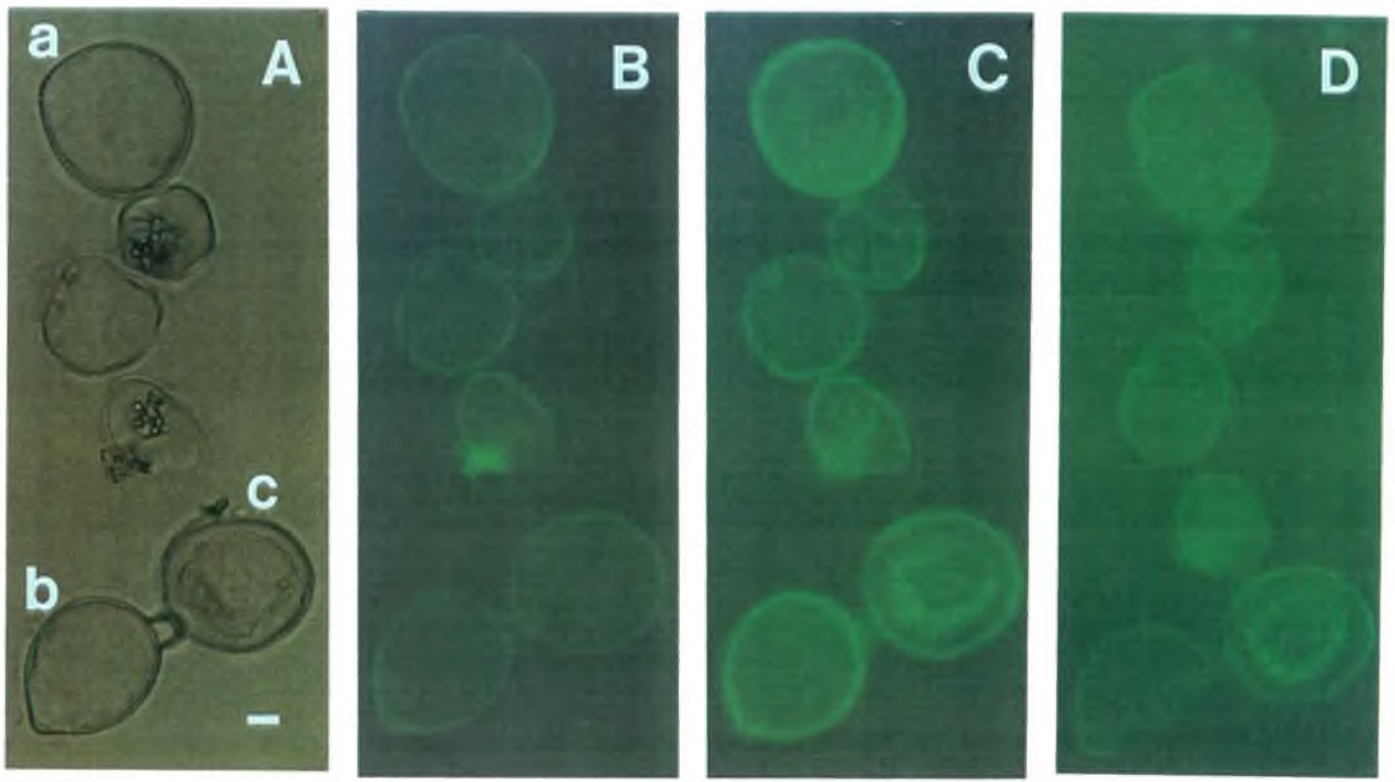

Fig. 5.3. Localization of fluo-3 AM in purple nutsedge suspension cells. The cells were incubated in 5 μM fluo-3 AM for 30 min in the dark at 24C. The extracellular dye was removed by spinning at 1,000 rpm for 5 min before observation with fluorescence microscopy. A. Bright-field image. Cells a and b were healthy with cytoplasm adjacent to the cell wall, and cell c appeared probably plasmolyzed. B. Distribution of fluorescence. The cell walls in all cells showed fluorescence mainly due to autofluorescence before adding ionomycin. C. Fluorescence image of cells 15 min after incubated with 10 μM ionomycin and 1 mM $CaCl_2$ in 10 μM HEPES buffer and 140 μM KCl, pH 8.5. The fluorescence appeared in the cytoplasm adjacent to the cell wall and cytoplasmic strands in the interior of cells. D. Fluorescence decreased in all cytoplasm regions but remained in the cytoplasmic strands of the plasmolyzed cytosol cell c 10 min after incubation with 1 mM $MnSO_4$ for 10 min. Bar = 10 μm.

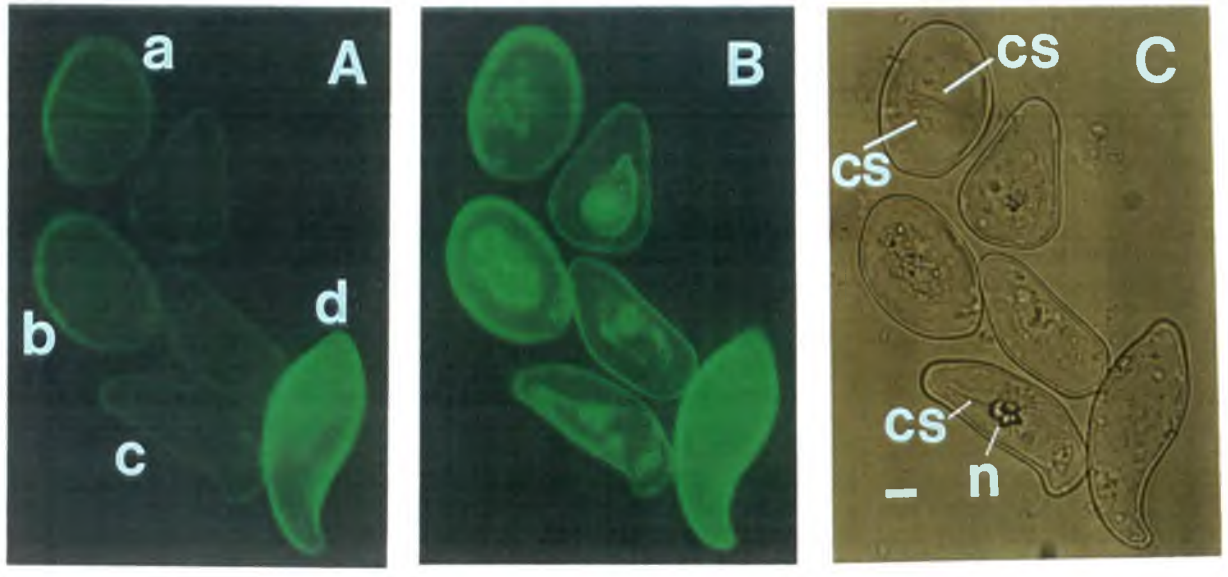

Fig. 5.4. Fluorescence microscopy images of $[Ca^{2+}]_i$ changes in purple nutsedge cells in response to elevated temperature. Cells were loaded with 5 µM fluo-3 AM for 30 min, then placed on the microscope slide. A. Fluorescence intensity $[Ca^{2+}]_i$ of cells at the resting level after 10 min incubation at 24C on the microscope slide. B. Fluorescence intensity $[Ca^{2+}]_i$ of cells after exposure to 35C for 30 min. C. Bright-field of the image B. cs = cytoplasmic strand, n = nucleus. Bar = 10 µm.

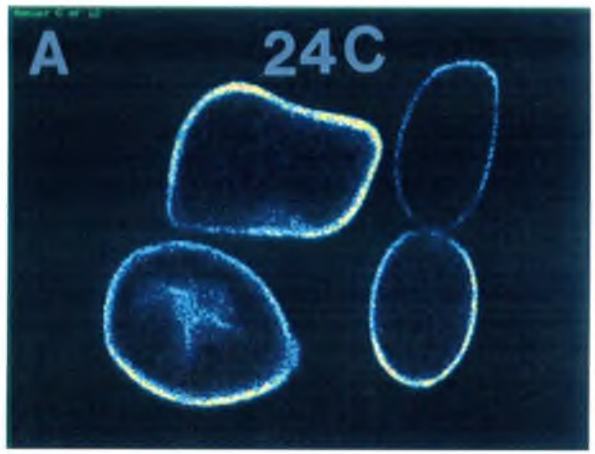

Fig. 5.5. Confocal laser scanning microscopy images of cytosolic calcium changes of suspension-cultured cells of purple nutsedge in response to heat pulse. Cells were loaded with 10 μM fluo-3 AM for 1 h. A. Images of the median plane of the cells at 24C before heat treatment. Fluorescence intensity values, indicating differences in Ca^{2+} content are represented by the color scale, where black is minimum and white is maximum fluorescence. B. Additive images of all 12 slices from the same cells as A. The cells were imaged at 24C before high temperature treatment (B1), after 10 min of 35C treatment (B2), after 30 min of 35C treatment (B3), and after 60 min of 35C treatment (B4). The color scale in B is the same as A. The fluorescence intensity was quantified by using relative pixel values (total pixel values over total pixel number in the area). Bar = 5 μm.

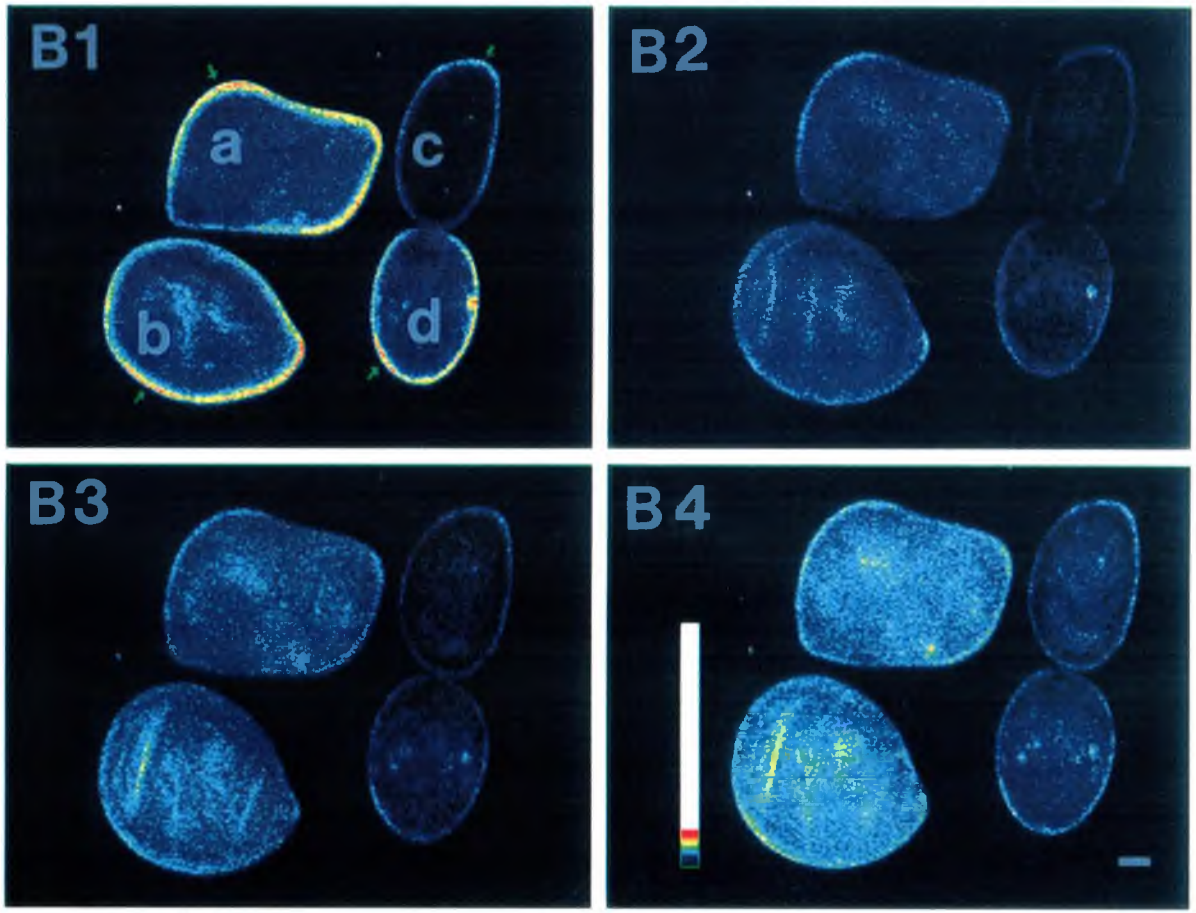

Fig. 5.5. (Continued) Confocal laser scanning microscopy images of cytosolic calcium changes of suspension-cultured cells of purple nutsedge in response to heat pulse.

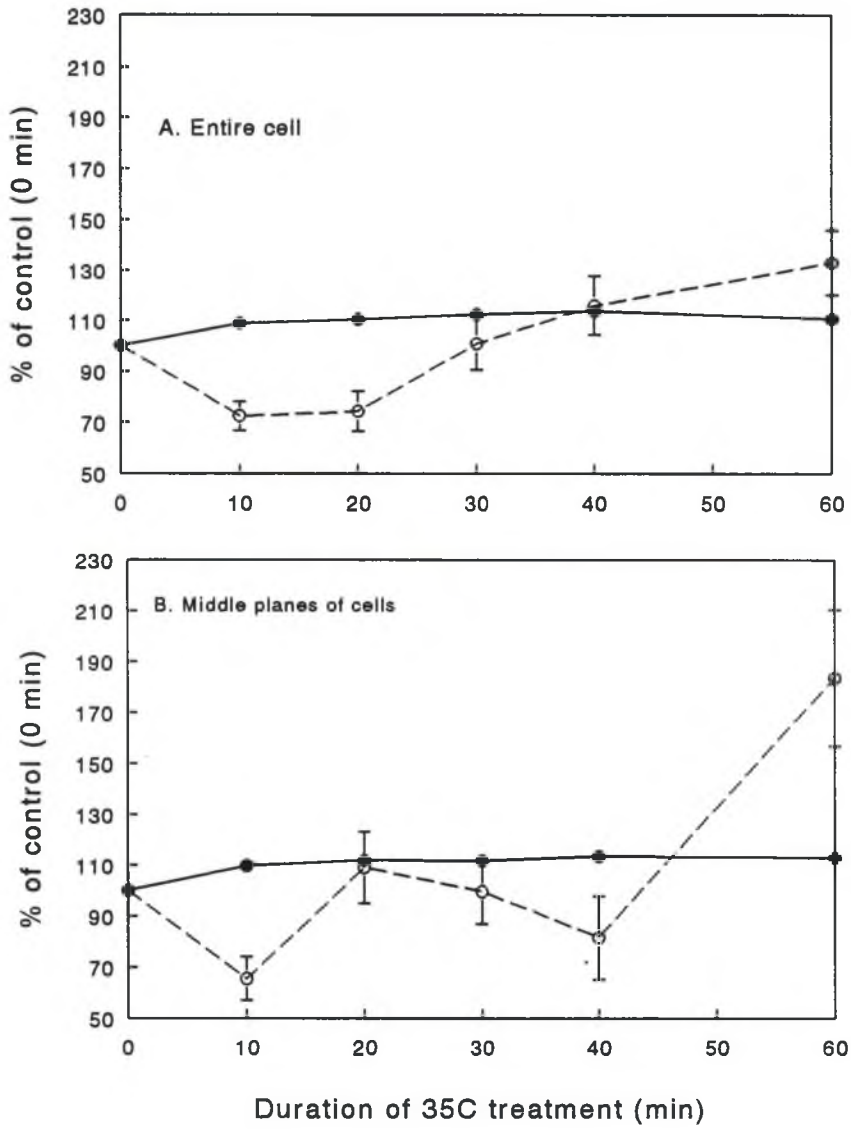

Fig. 5.6. Effect of heat pulse duration on the changes in sizes of cells (solid circle), amount of Ca^{2+} over a entire cell (open circle). The amount of Ca^{2+} in a entire cell was quantified by using total pixel values from the whole cell region. Results represent mean of relative values ± SE of four cells indicated in Fig. 5.5B.

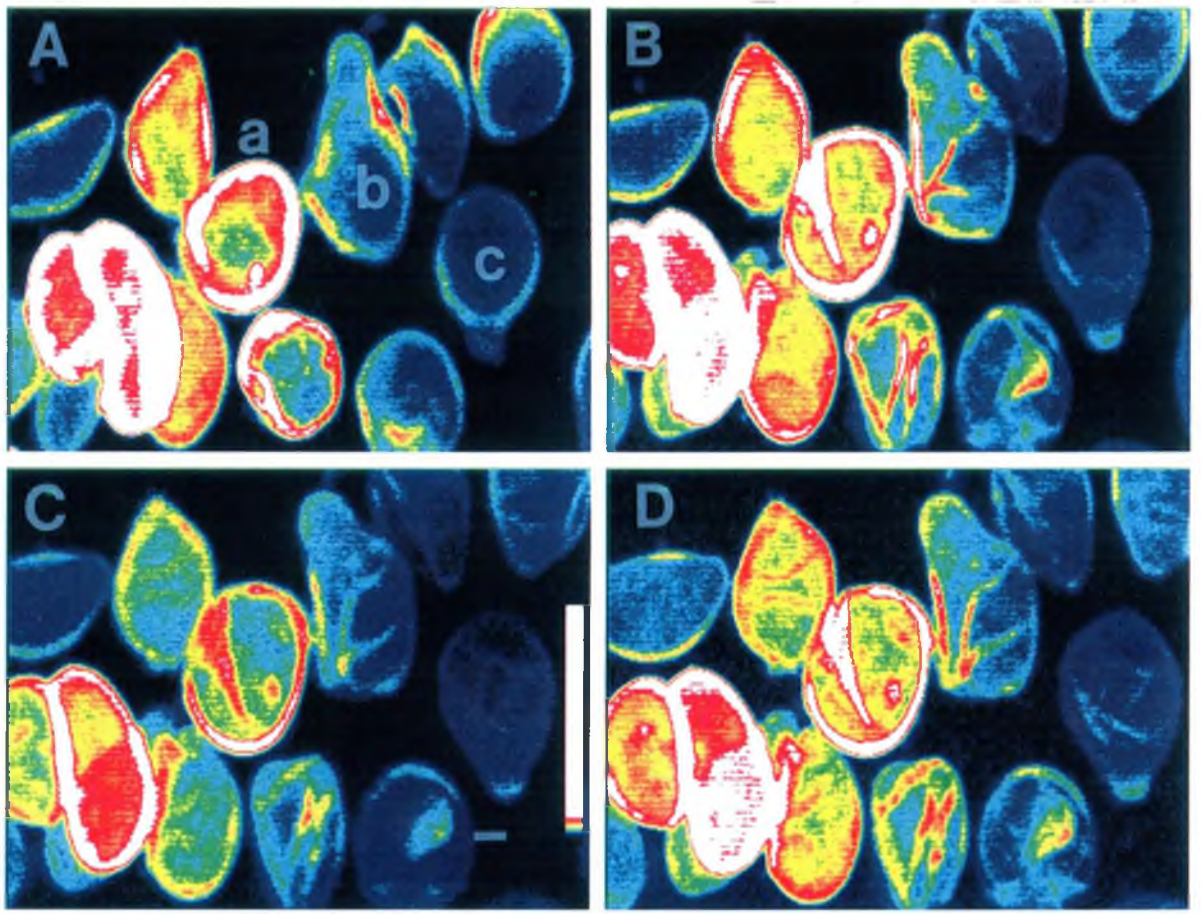

Fig. 5.7. Confocal laser scanning microscopy visualization of the kinetics of intracellular Ca^{2+} changes in response to a temperature fluctuation. Cells were loaded with 20 μM fluo-3 AM for 1 h. Images are of median plane of cells, 5 μm depth per plane. The duration of 35C treatment in images A, B, C, and D is 0, 30, 90, and 120 min respectively. Temperature at zero time was 20C. The corresponding cell temperatures are shown in Fig. 5.8B. Bar = 10 μm.

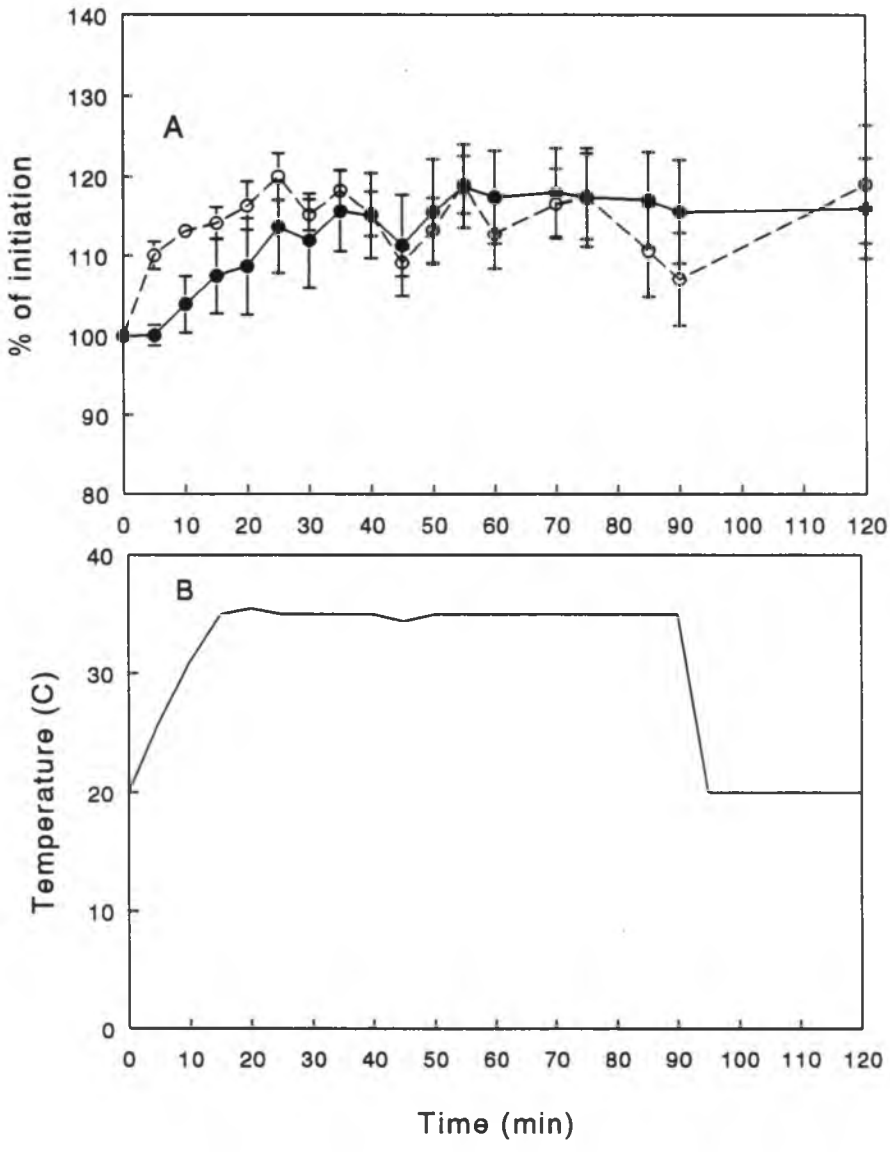

Fig. 5.8. Response of intracellular Ca^{2+} and cell size to withdrawal of heat stimulus. Temperature changes in a time course (B) is recorded from the surface of a thermal chamber. The corresponding changes of cell size (solid circle) and amount of intracellular Ca^{2+} (open circle) over an entire cell of a middle plate (A) are based on the image of Fig. 5.7. Data acquisition and image analysis were performed using the CoMOS program (Bio-Red). Results represent mean of relative values ± SE of three cells indicated in Fig. 5.7.

CHAPTER VI

THERMOPERIODICITY IN SHOOT ELONGATION OF PURPLE NUTSEDGE

Introduction

Thermoperiodicity or thermoperiodism refers to a physiological response of an organism to the alternation of warm and cool temperatures. The term thermoperiodicity in plants was coined by Went (1944). Went's definition and other researchers (Wellensiek, 1957; Dale, 1964; Friend and Helson, 1976) indicate that the thermoperiodic effect could stimulate or inhibit plant growth and development. The cause of these responses is due to temperatures alternations rather than differences in temperature itself. In addition, these temperature effects on seed germination frequently interact with light.

It is difficult to separate thermoperiodic effects from differences in temperature itself. Using constant upper or lower (Went, 1944), or mean temperature of the alternation cycle (Wellensiek, 1957; Haroon et al., 1972; Lionakis and Schwabe, 1984) as a reference temperature is not sufficient to demonstrate thermoperiodicity because the response of growth to constant temperatures is not linear over a wide range. The choice of suboptimal temperatures, or supraoptimal temperatures for their experiments can cause inconsistent effects over the wide range of temperatures (Friend and Helson, 1976). The plant is thermoperiodic (Dale, 1964) or optimally thermoperiodic (Friend and Helson, 1976) only if greater growth (dry weight, stem elongation, etc.) occurs under daily alternating temperature conditions than under the optimal constant temperature conditions of the same mean value. Accordingly a necessary criterion for thermoperiodicity is the occurrence of a remarkably different response at alternating temperatures than at an optimal constant, mean temperature. The working definition of thermoperiodicity in this study follows Dale's (1964).

Thermoperiodism is not a phenomenon common to all species. Little evidence for thermoperiodicity in developmental aspects of growth, (i.e., stem elongation, flower initiation) has been reported (Went and Sheps, 1969). Some papers provide good evidence for the absence of thermoperiodicity in fresh or dry weight accumulation in sugarbeet (Ulrich, 1952), *Phaseolus* (Dale, 1964), sugarcane (Glasziou et al., 1964), tomato (Hussey, 1965; Friend and Helson, 1976), tobacco (Haroon et al., 1972), and pea, bean, cucumber, corn, oats and wheat (Friend and Helson, 1976).

A systematic study on the effect of alternating temperatures on purple nutsedge (*Cyperus rotundus* L.) tuber sprouting has been reported (Miles et al., 1996). In their research, tubers with at least one shoot longer than 10 mm in length were assessed as sprouted. This parameter does not directly reflect growth by shoot extension. It was shown that tuber sprouting was greater under alternating temperatures (25/35C, 12/12h) in the dark than at a suboptimal constant mean temperature (30C). However, their experiments do not establish how shoot elongation responds to alternating temperatures, and whether the response is thermoperiodic. In these experiments, evidence was obtained on thermoperiodicity in shoot elongation. Some attributes of the alternating temperature function such as magnitude, upper and lower shifts in temperature, variations in the duration of lower and upper temperatures, and the number of fluctuating cycles were characterized.

Materials and Methods

Tuber sprouting and growth conditions. Glasshouse-grown unsprouted tubers were obtained as described in Chapter III. Tubers with budbreak that had at least one sprout 2 to 5 mm in length were incubated at constant 20 or 25C for at least 7 d before use in experiments. In an experiment to test the effective magnitude of alternating temperature

regimes, 24C was used as the mean temperature, and the tuber sprouts (2 to 5 mm in length) were incubated at constant 24C for 7d after budbreak at 20C. Sprouted tubers were incubated on two layers of filter paper moistened with 3.5 ml deionized water in a 100 x 15 mm Petri dish enclosed in a polyethylene bag to conserve moisture. The relative humidity in the incubator was kept at 60% to 70% .

Elongation measurements. The shoot length (accurate to 1 mm) from the bud base to the tip of scale leaf or primary leaf was measured at the end of 7 d. If one tuber possessed several shoots, the longest shoot was reported. The number of shoots longer than 10 mm per Petri dish was also recorded.

Temperature treatments. The sprouted tubers were subjected to various alternating temperatures. Programmable incubators (± 1.0C) were used for the daily alternating temperature regimes with equal duration (12 h) of upper or lower temperature, while the short period of temperature fluctuations were obtained by transferring tubers between incubators at designed temperatures for the required time intervals. It took ca. 30 min for the tuber's surface temperature to change from 20 to 35C or from 35 to 20C in the 20C and 35C incubators, and this time was included in the reported heating times. Tuber temperature changed much faster in incubators than in the soil. However, our preliminary data showed that the effect of rates of temperature change on shoot elongation was negligible.

In the first experiment, the optimal constant temperature for shoot elongation was determined in a preliminary trial by comparing shoot elongation under six constant temperatures ranging from 20 to 45C at intervals of 5C. The alternating temperature regime (30/40C, 12/12 h) was chosen to assess thermoperiodicity of shoot elongation, as the pre-determined optimal constant temperature was 35C.

In other experiments, constant temperature regimes were selected as the mean reference point to the alternating temperature regimes. In the experiment shown in Fig.

6.4, 25C was set as a base temperature for comparing the upward shifts from 25 to 35C and downward shifts from 25 to 15C in addition to constant mean temperatures of 30C and 20C. In the experiment of effect of duration of upper and lower temperture phases, sprouts preincubated at 25 and 30C were used for 23/30C and 30/40C alternating temperatures respectively. In the study on the number of temperature alternations, each cycle of temperature alternation contained 1h at 40C, and 23 h at 20C (referred to cyclic heat pulse); after temperature treatments, the tubers were maintained at constant 20C. The effect of cumulative heat of every warm pulse on the elongation was neglected due to the short duration of high temperature.

Statistical analysis. Each experiment was designed as a completely randomized design, consisting of four replicate dishes with 10 tubers per treatment. Each experiment was conducted at least twice. Results between experiments were similar, and data from one of the experiments is shown. Data were subjected to analysis of variance (ANOVA) to determine sources of variation and provide the estimates for calculating the standard errors of means (SE). Temperature responses of tuber sprouts to the number of pulses was analyzed by linear and/or nonlinear regression. Duncan's multiple range test was used to determine whether treatments were significantly different.

Results

Thermoperiodic effect. The optimal constant temperature for shoot elongation of purple nutsedge was 35C (Fig. 6.1), and the low and high constant temperature thresholds were about 25 and 40C respectively. Shoot elongation under alternating temperature of 30C for 12 h and 40C for 12 h, however was 2.3-fold greater than at the 35C optimal constant temperature, and 97.5% of the shoots were longer than 10 mm. Under constant temperature of 20 and 25C, the tuber sprouts had no extension after 7-d incubation. Over

the range 25 to 35C, shoot elongation increased linearly. When temperature increased above 35C, the shoot extension was markedly reduced. The shoot length at the peak temperature 35C was 16 mm, and only 50% of the shoots were longer than 10 mm. Sprouted tubers exposed to constant temperature of 40 and 45C had the same level of shoot elongation as 20 and 25C. These results indicate that growth as measured by shoot elongation of purple nutsedge, was thermoperiodic.

Effective magnitude of alternating temperature regimes. Shoot length increased to more than 8.5 times at alternating temperatures of 20/28C and 18/30C compared with the constant mean temperature, and 88% and 98% of the shoots were longer than 10 mm (Fig. 6.2). At the alternating temperature regimes investigated, temperature differential below 4C degree did not significantly promote shoot extension within 7 d. No significant increase in shoot length occurred at alternating temperatures of 23/25C and 22/26C, with only 28% of the shoots were longer than 10 mm. The response of the number of shoot longer than 10 mm to varying magnitudes of alternating temperatures coincided with shoot elongation (Fig. 6.2).

Effect of duration of upper and lower temperature phases. Shoot length increased to maximum only with 1 h of upward shifts from 30 to 40C. The stimulation was less when the daily duration of 40C upper temperature was greater than 6 h (Fig. 6.3). In the 20/30C alternating temperature regime, shoot length was 62% of maximum with a 1 h upward shifts from 20 to 30C, and 91% of maximum at 3 h at 30C (Fig. 6.3). A similar stimulatory effect was observed in other experiments (Fig. 6.4, 6.5) with 1 h at the upper temperature with 7 cycles of fluctuating temperature regimes. Tuber sprouts exposed to 25/35C (23/1 h), and 20/40C (23/1 h) provided 72.7% (Fig. 6.4) and 70% (Fig. 6.5) of shoot elongation as the corresponding 12-h alternating cycles respectively. In contrast, shoot elongation with 1 h of daily downward shifts from 40 to 30C or from 30 to 20C was less than 50% of the maximum obtained by 21 h or 9 h of downward shifts respectively;

shoot elongation gradually increased with increasing duration of the lower temperature phase (Fig. 6.3). Fluctuating temperatures were ineffective when given 1 h at 15C (lower phase temperature) followed by 23 h at 25C, but promoted shoot elongation slightly when given 12 h at 15C, followed by 12 h at 25C (Fig. 6.4).

Dependence on the number of temperature fluctuating cycles. To test the necessity of multiple cycles of temperature fluctuations for shoot elongation, sprouted tubers were exposed to various cycles of 1 h at 40C heat pulse with 23 h at 20C cool phase, and maintained at constant 20C after the designed number of fluctuating cycles. The relationship between the number of fluctuating cycles and shoot elongation was best fitted as a sigmoidal curve ($r^2 = 0.97$). A single 40C pulse had little contribution to elongation when compared to a constant 20C (Fig. 6.5); only 18% of the shoots were longer than 10 mm. Shoot elongation increased linearly with increasing temperature fluctuations of up to three cycles, then increased at a lower rate (Fig. 6.5). After five cycles, shoot elongation only slowly increased. This result suggested that multiple high temperature pulses were required for the stimulation of shoot elongation, and the effect of fluctuating cycles on shoot elongation was additive in a certain range.

Discussion

Thermoperiodicity in shoot elongation of purple nutsedge was determined. Shoot elongation was much greater under an alternating temperature regime 30/40C, 12/12 h than at optimal constant mean temperature 35C (Fig. 6.1). This heat-stimulatory effect was also obtained with other suboptimal temperature regimes. Alternating temperature (25/35C, 12/12 h) provided greater elongation than the same mean, constant temperature 30C (Fig. 6.4), and the extent of elongation was even greater than the 30/40C alternating temperature that corresponded with the optimal 35C constant temperature. The

stimulatory effect also occurred under 15/25C, 12/12C alternating temperature regime which was at the low constant temperature threshold. The fact that the thermal response of shoot elongation relies on the magnitude (Fig. 6.2) and the number of cycles of temperature fluctuations (Fig. 6.5) indicates an alternating temperature-dependent response. Thus temperature itself was not crucial, provided the temperature difference between the upper and lower phases was adequate. The response of shoot elongation in purple nutsedge can be classified as thermoperiodic because the response met the established criteria (Went, 1944; Dale, 1964; Friend and Helson, 1976).

Only 1 h at 40C at the end of a 23 h at 30C treatment was sufficient to obtain a maximum level of shoot elongation, while shoot elongation linearly increased as the duration at lower phase temperature increased over the range from 1 to 21 h. It appears that the response to the upward shifts acts as a quick reaction, while the downward shifts act as a slow reaction. The necessity of multiple cycles of temperature fluctuations indicates that both upward and downward shifts may work in concert. In the germination of upper cocklebur seeds, it was suggested that the cool phase acted mainly in the production and accumulation of ATP by activating the alternative path way during the subsequent warm phase, while the warm phase supplied ADP and especially, AMP (Esashi et al., 1983). Consequently the size of the adenylate pool and the energy charge increased with an increasing number of sequential temperature-alternating cycles (Esashi et al., 1983). Such a collaborative pattern may occur in purple nutsedge.

The process of sprouting of purple nutsedge tubers can be separated into two subsequent steps: budbreak and shoot elongation (Nishimoto et al., 1995). Both of them are thermal regulated, and require a temperature differential for the particular functions. For budbreak, only the upward shift in a fluctuating temperature regime was effective; a single warm pulse was sufficient to terminate dormancy, and cause bud emergence. Thus the response of budbreak to higher temperature is likely a trigger action (Chapter III). In

contrast, shoot elongation relies on both upward and downward shifts. In addition, a number of continual thermoperiodic cycles was required to promote elongation, and the effect of each cycle was additive. Thus the temperature response of shoot elongation has a rhythmic action.

The requirement for multiple cycles of fluctuating temperature to cause elongation of purple nutsedge may be of importance in its survival. Dormant tubers of purple nutsedge may be accidentally exposed to higher temperature, and budbreak occurs. If the temperature remains cool, the sprouts will not elongate. The sprouts will continue to elongate and form shoots and leaves, when the temperature is warm enough with adequate temperature differential between the day and night. Favorable conditions occur when a plant canopy above the soil surface is removed (Miles et al., 1996), where purple nutsedge can grow without competition with other plants. This double check mechanism would aid purple nutsedge in sensing favorable or adverse environmental conditions. In consideration of this mechanism, for the purpose of inducing all the tubers to sprout and form leaves, strategies to induce a single brief increase in soil temperature may not prove effective, as a number of temperature fluctuation cycles are necessary to cause budbreak and subsequent shoot elongation. One strategy to induce these temperature fluctuating cycles is long-term soil solarization (Miles, 1991).

The number of shoots longer than 10 mm increased by elevating the magnitude of alternating temperatures. This agrees with the finding of Miles et al. (1996). Only 10% of shoots longer than 10 mm were obtained under conditions of 4C temperature differential and 24C daily mean temperature in 7d (Fig. 2). The magnitude of alternating temperature is an important factor in application of soil solarization to increase sprouting of purple nutsedge (Miles, 1991; Miles et al., 1996). The mean daily soil temperature at 15 cm depth ranges from 22 to 27C in Hawaii, and the daily difference between minimum and maximum was less than 2C. Under soil solarization, the mean daily soil temperature at 15

cm depth increased to 5 to 9C, and the daily difference between minimum and maximum is ca. 4 to 5C, and would induce 94% to 100% of shoots to elongate longer than 10 mm (Miles, 1991). The explanation for such a large improvement could be that the effective temperature magnitude for budbreak or shoot elongation is dependent on alternating temperature regimes; as the mean temperature is increased, the minimum magnitude decreases, e.g., when the mean daily soil temperature is ca. 30C, a 4C temperature differential is enough to cause shoot elongation.

Information gained from this study would aid in understanding the dormancy mechanism of purple nutsedge, that could lead to management practices to induce shoot elongation. In addition, evidence for thermoperiodicity in purple nutsedge shoot elongation provides a potential model for studying the mechanism of alternating temperature effects.

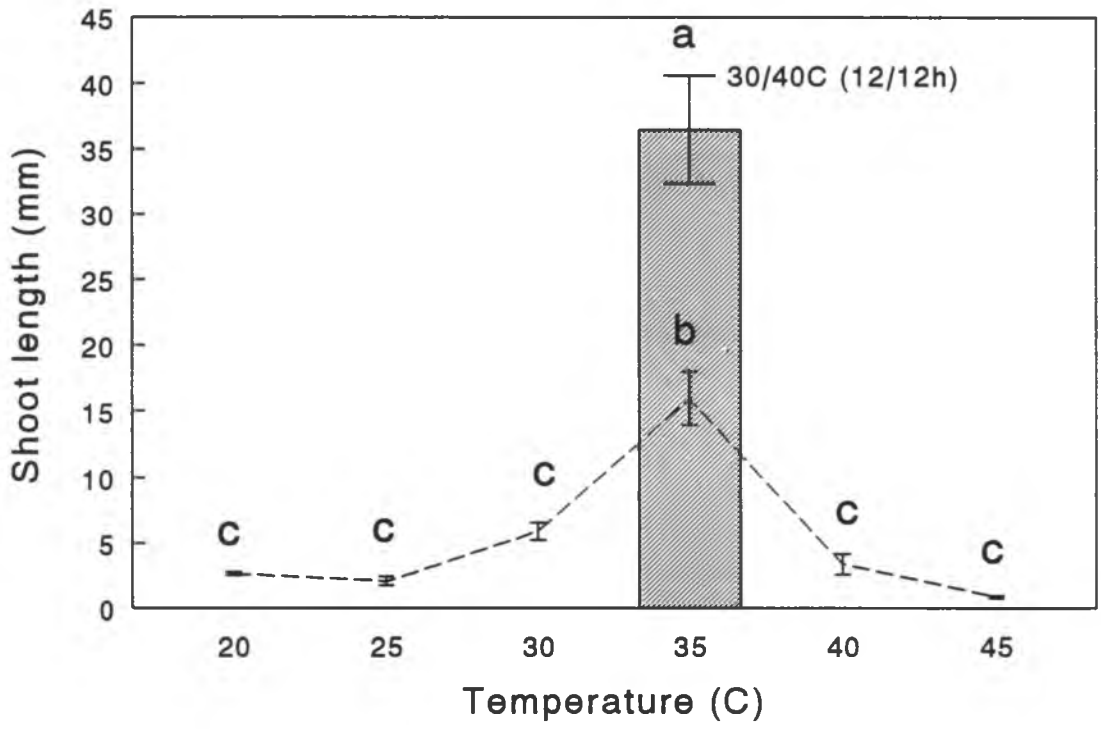

Fig. 6.1. Response of purple nutsedge shoot elongation to constant temperatures and an alternating temperature regime (30/40C, 12/12 h). The response of shoot elongation to constant temperatures was depicted by the line graph, and the alternating temperature regime with 12 h at 30C and 12 h at 40C by the vertical bar to compare with its mean, optimal constant temperature (35C). Data points with different letters differ significantly by Duncan's multiple range test, $P = 0.05$.

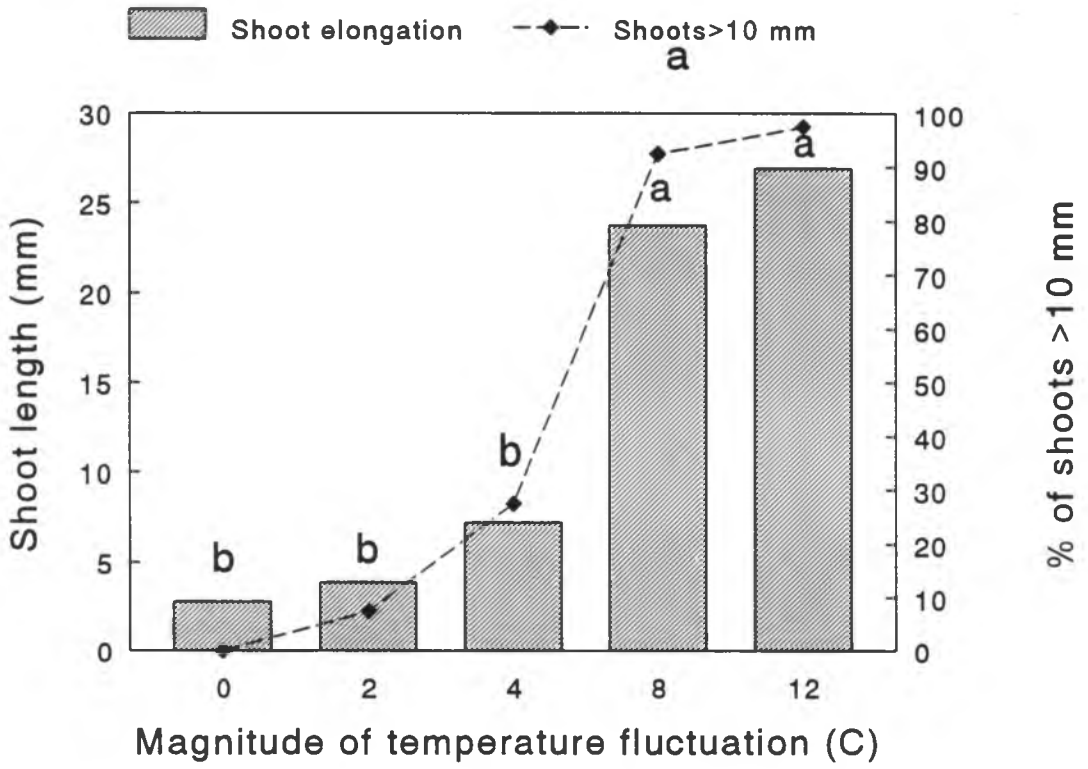

Fig. 6.2. Effect of magnitude of alternating temperatures on purple nutsedge shoot elongation. The value for shoot elongation at constant 24C is at zero magnitude. Each alternating temperature with 12-h warm phase and 12-h cool phase is symmetrical around a mean temperature of 24C. The magnitude is the difference between the peak and trough temperatures. Bars with different letters differ significantly by Duncan's multiple range test, $P = 0.05$. The mean separation of shoot length and the number of shoots larger than 10 mm to a certain alternating temperature is identical, so bar and line graphs share the same letters.

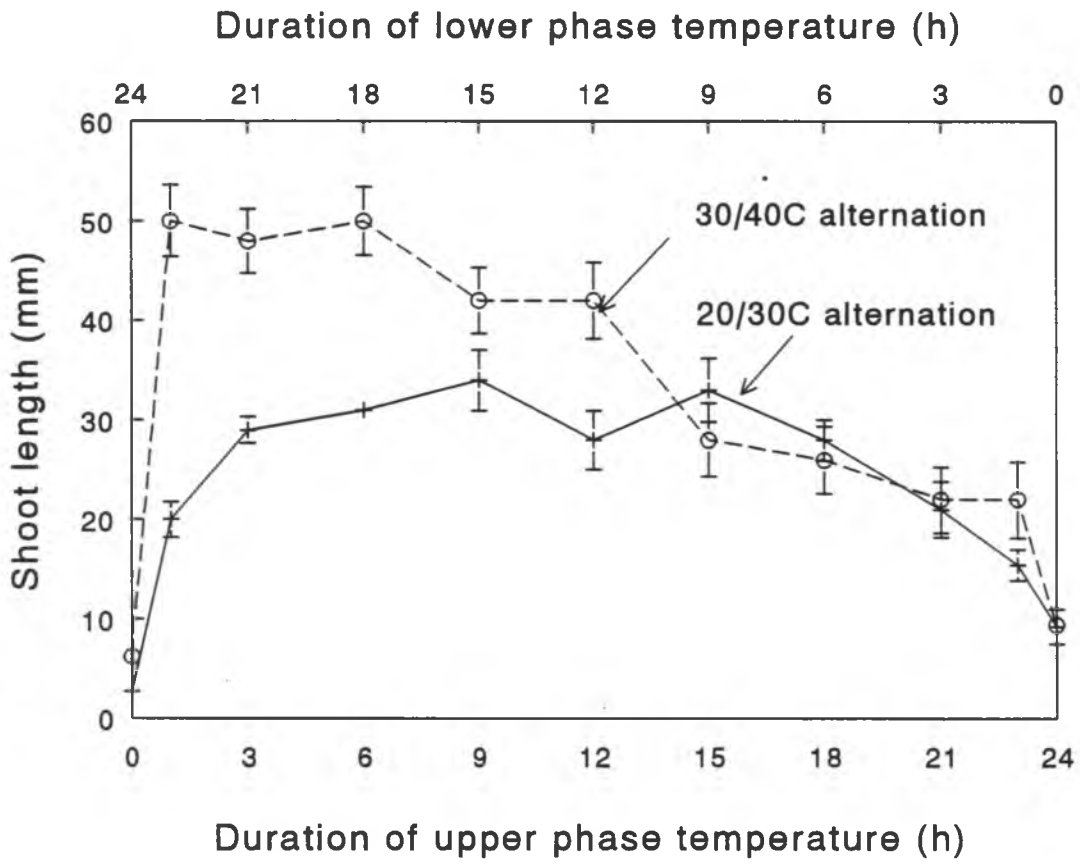

Fig. 6.3. Comparison of shoot elongation in response to variations in duration of upper and lower temperature phases in the alternating temperature regimes (20/30C and 30/40C, 12/12 h) with 7 cycles. Tuber sprouts were exposed to temperature shifts from lower to higher (see lower x axis) or shifts from higher to lower (see upper x axis) for various times, and returned to the base temperature for the remainder of the 24-h cycle. After 7 cycles, the cumulative shoot length was determined. The values for shoot elongation at constant lower and higher temperatures are at 0 and 24 h respectively. Observations with the same value are hidden from view.

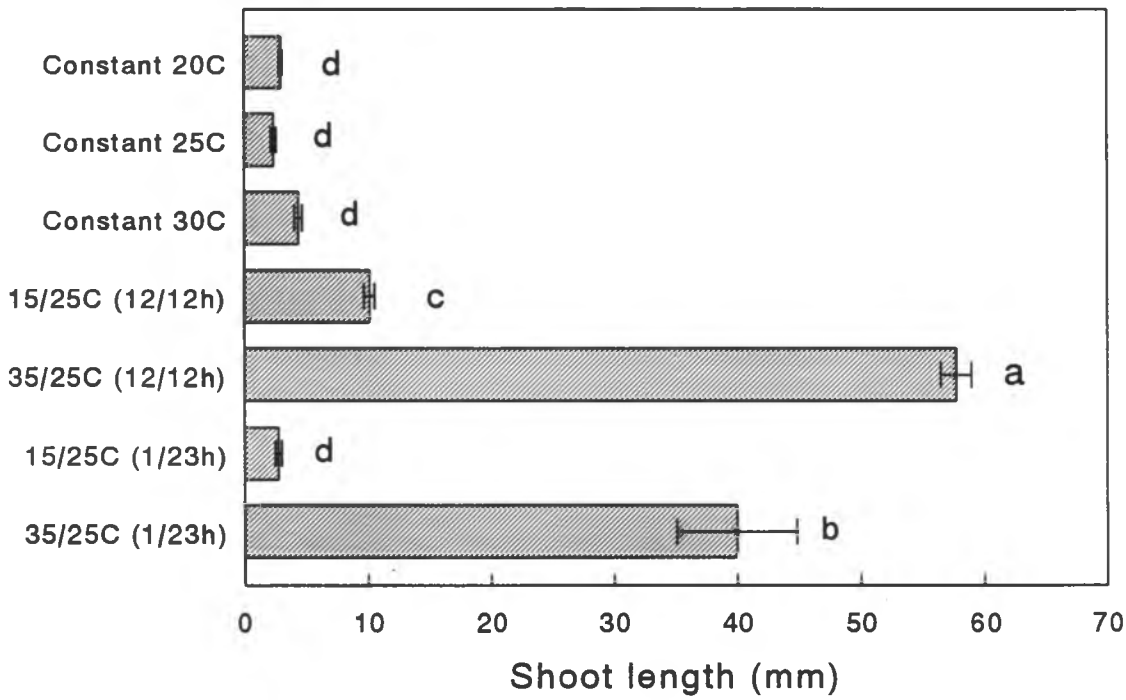

Fig. 6.4. Thermoperiodic shoot elongation at suboptimal and low temperature threshold regimes. The mean temperatures of alternating temperature 15/25C and (35/25C) are at suboptimal and low temperature threshold ranges respectively, each alternating temperature cycle has two sets of shifting times i.e., 12/12 h, and 1/23 h. Constant 20, and 30C treatments were mean temperatures for the two equal-duration alternating temperature cycles; constant 25C was set as a base temperature for the two unequal-duration fluctuating temperatures. Bars with different letters differ significantly by Duncan's multiple range test, $P = 0.05$

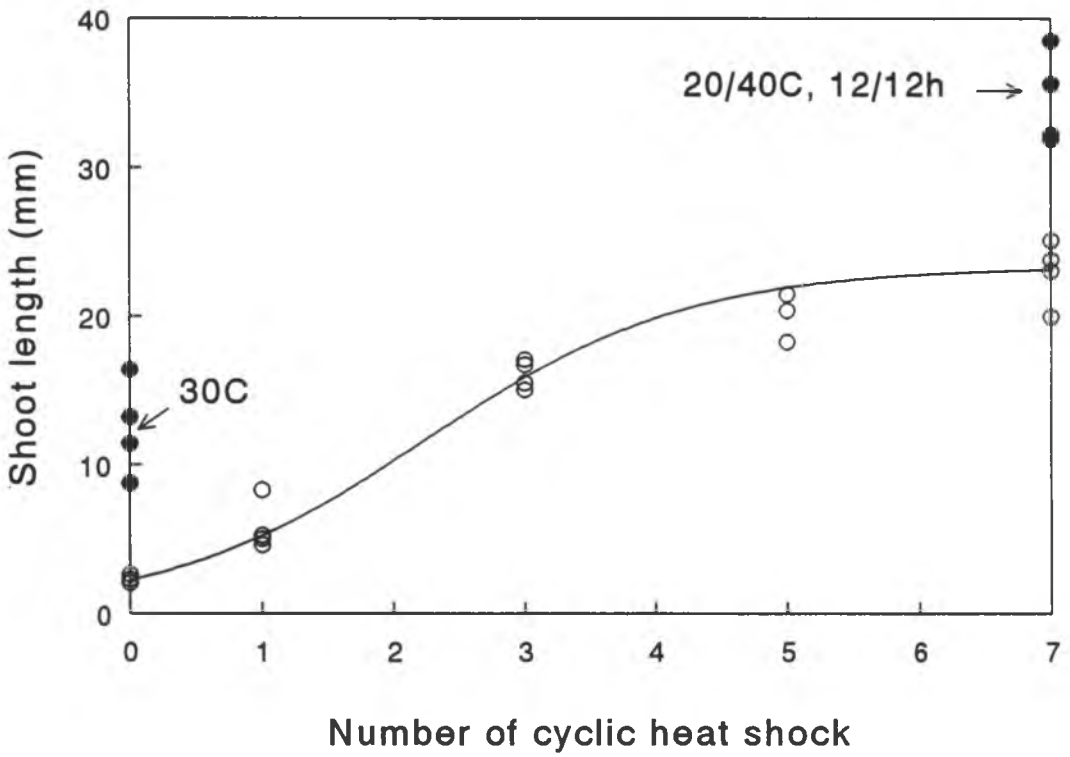

Fig. 6.5. Response of shoot elongation to the number of fluctuating cycles. One high temperature pulse (40C for 1 h) was inserted after 23-h incubation at 20C in each fluctuating cycle. Tuber sprouts were incubated at 20C after exposure to the indicated number of fluctuating cycle(s). The fitted model is $Y = -5.8500 + 29.0566/(1 + \exp(-(x-1.4959)/1.5069)$; $r^2 = 0.97$.

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

It has been established that tuber dormancy is the most important mechanism by which purple nutsedge, recognized as the world's worst weed (Holm et al., 1977), persists in the environment, and alternating temperatures stimulate sprouting of purple nutsedge tubers (Miles, 1991; Miles et al., 1996). However, the mechanism for dormancy release by temperature is poorly understood. This study approached the mechanism by characterizing the physiological responses of purple nutsedge tuber buds to fluctuating temperatures, particularly focusing on calcium as a messenger in coupling external heat signal and cellular and morphogenesis of sprouting of purple nutsedge tubers.

The experimental approach contained two steps. The first was to observe whether the effect of alternating temperatures on the tuber sprouting was rapid or slow. The second was to determine if Ca^{2+} was the second messenger in the thermal signal transduction by obtaining indirect evidence by pharmacology, and then direct evidence by visualization with LSCM or epifluorescence microscopy. To facilitate the study, sprouting was separated into two processes: budbreak and shoot elongation, and fluctuating temperature was defined as a wave-changing temperature including daily alternating temperature and single warm pulse or heat pulse. The selection of " dormant tubers" by pre-incubating unsprouted tubers at a base temperature (20C) for 2 weeks was crucial to the experiments.

Three experimental systems were used. The first was the use of whole tubers to determine the responses of budbreak and shoot elongation to temperature treatments. The second was an excised bud system for pharmacological studies. The third was suspension-cultured cells to visualize cytosolic calcium changes in response to heat pulse.

The effect of fluctuating temperature on budbreak of purple nutsedge tuber was characterized. Dormant tubers exposed to 35C for 30 min to 12 h, then 20C for 6.5 d had

80% to 92% budbreak. A 15C increase above 20C in 3 minutes also brought about substantially more budbreak than those without the heat treatment. The budbreak at 35C for 12 h was similar to that obtained with seven cycles of 20/35C (12/12 h). As the number of 30 min exposures to 35C increased, budbreak did not significantly increase. A change in temperature (from 25 to 15C for 12 h) did not promote budbreak. Exposure to temperatures 10 to 20C above a base temperature of 20C stimulated a similar amount of budbreak. Varying the rates (0.02 to 0.5C per min) of a single temperature shift from 20 to 35C did not effect budbreak. The results suggested that budbreak of purple nutsedge tubers responds to warm temperature as a trigger action.

The role of Ca^{2+} in budbreak was investigated by facilitating or artificially blocking the influx of cytosolic Ca^{2+} in an excised bud system. Individual buds were excised from unsprouted tubers grown in the glasshouse. A single heat pulse (35C for 1 h) stimulated budbreak of excised buds. This induction was substituted by a Ca^{2+} ionophore, ionomycin, and reversed by a Ca^{2+} chelator, EGTA. Plasma membrane Ca^{2+} channel blockers, verapamil and diltiazem slightly reduced the heat-stimulatory effect. The effects of the promoter and the inhibitors were dose-dependent. These data suggest that Ca^{2+} may be involved in heat pulse-inducible budbreak of purple nutsedge tubers, possibly acting as a messenger in the thermal signal transduction pathway.

Suspension-cultured cells were developed from the shoot tip of purple nutsedge. Using fluorescence and confocal imaging of Ca^{2+} in the cells loaded with fluo-3 AM, the role of Ca^{2+} in the thermal signal transduction pathway was further investigated. With increasing temperature from 20 to 35C, cell expansion initially occurred within 5 min. Coincidentally intracellular Ca^{2+} content ($[Ca^{2+}]_i$) was either reduced or increased and re-distributed, and then steadily increased. Irregular oscillations in $[Ca^{2+}]_i$ was observed in the middle optical plane of cells. The elevation of intracellular Ca^{2+} signal was accompanied by the appearance and extension of cytoplasmic strands, indicating that the

formation of cytoplasmic strands may be correlated with Ca^{2+} influx. A downward temperature shift from 35 to 20C did not cause a reduction of the Ca^{2+} signal within 30 min. These data provide the first direct evidence for the $[Ca^{2+}]_i$ elevation in purple nutsedge cells as a result of heat pulse induction, and suggested that $[Ca^{2+}]_i$ might play a role in the promotion of cell division.

Thermoperiodicity in shoot elongation of purple nutsedge was determined by comparing growth at optimal constant temperature with alternating temperatures. Tuber sprouts exposed to alternating temperatures with 12-h 30C and 12-h 40C increased shoot elongation by 2.3 times as compared with the optimal mean of 35C. Temperature differences of 2 and 4C in alternating cycle around the mean 24C had little effect on growth; 8C provided significant shoot elongation. The growth response was more sensitive to the upper temperature phase than the lower phase. Shoot elongation increased with the number of temperature fluctuating cycles. The evidence for thermoperiodicity in purple nutsedge shoot elongation provides a potential model for studying the mechanism of alternating temperature effects, and a physiological basis for a practical approach of long-term soil solarization to induce tuber sprouting. These data suggest a double check mechanism that would aid purple nutsedge in sensing favorable or adverse environmental conditions. This insight may be of importance in developing management practices to induce sprouting. In consideration of this mechanism, for the purpose of inducing all the tubers to sprout and form leaves, strategies to induce a single brief increase in soil temperature will not prove effective; a number of temperature fluctuation cycles will be necessary to cause budbreak and subsequent shoot elongation. Long-term soil solarization may be a strategy that causes these temperature fluctuating cycles (Miles, 1991).

Future efforts should focus on the identification of the Ca^{2+} target in purple nutsedge cell to accomplish the demonstration of Ca^{2+} as a messenger in thermal signal transduction pathway. To do so, the subcellular localization of Ca^{2+} changes, and quantitative determination of Ca^{2+} changes are required. The elucidation of the relationship between the Ca^{2+} signal and actin filament or microtubule will provide insight on whether the Ca^{2+}-activated, actin-binding protein, gelsolin, is a Ca^{2+} target protein. Development of monoclonal antibodies for gelsolin and Ca^{2+}-binding gelsolin will facilitate the demonstration of gelsolin participation in the formation of cytoskeleton-associated cytoplasmic strands. On the other hand, the determination of Ca^{2+} changes of bud tissues (presumably meristematic cells) in response to high temperature is of importance in linking the heat signal and budbreak response. In addition, establishing biochemical markers or parameters of heat pulse-induced budbreak are essential to further identify the heat-budbreak coupling pathway, and eventually explain the specificity of cellular responses triggered by Ca^{2+}. In terms of thermoperiodicity, it is of interest to determine the existence of circadian rhythms of shoot elongation and corresponding biochemical or molecular markers or parameters.

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